CLONAL PROPAGATION OF PHALAENOPSIS

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By

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ABSTRACT

Phalaenopsis was clonally propagated by use of in vivo and in vitro methods. In vivo, plantlets formed naturally on the node and tip of inflorescence, or root. Application of N-6-benzyl adenine to exposed buds on the inflorescence spike to induce plantlet formation was not very successful.

Rapid clonal propagation was successfully accomplished by use of in vitro culture techniques. Explants from the nodal buds of inflorescence were the most suitable material for culture, although apical and axillary buds from the stem could also be used. When basal nodes from inflorescences after flowering or young inflorescences were cultured in basal media (BM = Vacin and Went + 15% coconut water), one to four plantlets rather than protocorm-like bodies (plbs) were obtained from a single node. In order to produce more plantlets other plant parts such as leaf, stem and root were separated and cultured in various media. Plantlets were produced from stem and leaf cultures in Vacin and Went with 50% coconut water without sucrose (VW + 50%CW-Su). When stems and roots were cultured in basal medium + 1 ppm 2,4-dichlorophenoxyacetic acid (BM + 1 ppm 2,4-D) tumors were produced. The origin of tumor was endogenous. Protocorm-like bodies (plbs) and plantlets were produced on tumors when 2,4-D and sucrose were omitted from the medium.

Upon culturing shoot tips in constantly agitated liquid BM for one month and transfer to agar medium for another month, plbs were produced. An indefinite number of plantlets could be obtained through method that produce plbs. To promote multiplication and differentiation of plbs into plantlets sucrose was removed from the medium. Then for optimum

growth of plantlets, sucrose was again added to the medium.

Subculture of plbs at one leaf stage resulted in production of new plbs on the original plb and on young leaf. Usually a cell in the epidermis divided anticlinally forming two protruding cells. Further divisions of these meristematic cells formed a globular plb. Cells on one side of the globular structure enlarged while cells in the other portion remained meristematic. This meristematic area organized a shoot tip before root initial.

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INTRODUCTION

Orchids are propagated by two methods: sexual and asexual. In sexual propagation seeds are germinated on artificial media under aseptic conditions. Since most orchid hybrids are highly heterozygous, it is difficult to obtain a great number of uniform plants from seedling populations. Vegetative or asexual propagation is accomplished most commonly by division or cuttings, but increasing a cultivar by this method is extremely slow.

In the case of some Phalaenopsis, "topping" the main shoot provides a cutting and on the remaining basal stump axillary shoots may develop to provide a few more plantlets. In some species (Phal. equestris, Phal. intermedia, and Phal. schillerana), plantlets develop spontaneously at the tip of the inflorescence after flowering. In other species (Phal. lueddemanniana) plantlets form on nodes of the inflorescences. In the case of Phal. stuartiana and some of its hybrids, plantlets may form on the roots. (Northen, 1970).

Inflorescences of Phalaenopsis which emerge from the axil of the leaves are long in most species. The upper nodes bear flowers while the lower nodes have dormant buds. If the inflorescence is cut just below the node that produced the first flower, spikelets may develop from the upper nodes, producing a second spray of flowers. The lower nodes have dormant buds which rarely develop. A technique (Rotor, 1949) was, therefore, developed to induce plantlets from the dormant buds on the lower nodes by placing node cuttings from inflorescences into sterile medium. However, this technique, although attempted by many commercial growers and hobbyists, was abandoned earlier owing to the high percentage

of contamination until it was modified by Sagawa and Niimoto (1960). Further modifications have been made by Sagawa (1961), Urata and Iwanaga (1965), Kotomori and Murashige (1965), Scully (1965, 1966), Tse et al. (1971), and Intuwong et al. (1972) which have increased the success of this method for propagation of Phalaenopsis.

Recently tissue culture methods have been more commonly utilized as a means for vegetative propagation of orchids. The procedure by which a shoot tip containing a meristem and several leaf primordia is excised and placed in culture where it develops protocorm-like bodies (plbs), and eventually plantlets was developed by Morel (1960), and is called meristem culture (shoot tip culture). Clonal propagation by shoot tip culture is now being employed on a commercial scale with Cymbidium, Dendrobium, Cattleya, and Miltonia (Morel, 1960, 1964; Wimber, 1963, 1965; Sagawa et al., 1966, 1967; Bertsch, 1967; Reinert and Mohr, 1967; Scully, 1967; Lindemann et al., 1970; Werckmeister, 1971; Fonnesbech, 1972a, 1972b). Other genera of sympodial orchids which have been successfully propagated by meristem culture include Calanthe, Odontonia, Vuvstekeara, Spathoglottis, Cyrtopodium, Lycaste, Phaius, Oncidium and Zygopetalum (Morel, 1965, 1971; Vacherot, 1966; Fast, 1973). More recently, monopodial orchids such as Rhynchostylis (Vajrabhaya and Vajrabhaya, 1970), terete-leafed vanda (Kunisaki et al., 1972), strap-leafed vanda (Teo et al., 1973) and Sarcanthine orchids (Intuwong, 1973) have also been propagated successfully by use of shoot tip culture technique. Unfortunately, not all genera respond as readily to this technique; Paphiopedilum and Phalaenopsis fall in this category.

Initially shoot tips were the sole source of explants for tissue culture. However, practically all parts of the orchid plant -- root tips of Dendrobium (Kim, 1973), leaf tips of Laeliocattleya and Epidendrum (Churchill et al., 1970, 1971, 1973), young leaf of Cattleya seedlings (Champagnat, 1969, 1970) and young inflorescence of Ascofinetia (Intuwong and Sagawa, 1973) -- have now been induced to form plbs and plantlets.

Among orchids Phalaenopsis is popular both as a potted plant and as a cut flowers for use in bouquets and corsages. However, the limited number of clones currently available one mericlone (Phalaenopsis Capitola 'Moonlight'), 12 clones in limited numbers produced by stem propagation and 15 clones in limited quantity produced by a 'solid state process' indicates that Phalaenopsis is difficult to propagate asexually. Therefore, the purpose of this study was to develop more efficient in vivo and in vitro methods for rapid clonal propagation of Phalaenopsis with the application of growth regulators to dormant buds on inflorescences, improved aseptic organ culture and modifications of culture media. Anatomical and morphological studies were used to determine the mode of origin of plantlets.

MATERIALS AND METHODS

Plants used in this study were Phalaenopsis and related hybrids in the collection of the Horticulture Department, College of Tropical Agriculture, University of Hawaii, and are listed in Table I.

Since there were a substantial number of plants of D. pulcherrima, formerly known as Phal. esmeralda and Phal. buyssoniana in the collection, it was used in this study.

IN VIVO

N-6-benzyl adenine (BA) in lanolin paste was prepared by the method of Thompson and Jacobs (1966) incorporating 500, 1000 and 2000 ppm and applied by use of a glass rod.

IN VITRO

Sources of explants used in this study were shoot tips (terminal and axillary buds), nodes, and tips of inflorescences. The shoot tip culture method used was as described by Intuwong (1972). Nodes from mature and young inflorescences were cultured by the method described by Intuwong et al. (1972).

For organ culture, plantlets obtained from inflorescence node culture were used. Leaves, stems and roots were excised and grown separately.

Basal medium used was modified Vacin and Went medium (Sagawa et al., 1966) adjusted to pH 5.0 before autoclaving in both liquid and solid form. For solid medium 0.9% agar was added.

The basal medium was modified further by adding N-6-benzyl adenine (BA) at 0, 1, 5, 10 and 20 ppm; α-naphthaleneacetic acid (NAA) at 0, 1, 5 and 10 ppm for inflorescence node and bud propagation; and

2,4-dichlorophenoxyacetic acid (2,4-D) at 0, 0.5, 1, 2, 3 and 4 ppm for organ culture.

For media studies to determine the sequential requirement for the various stages of growth, basal medium with varying concentrations of coconut milk (0, 15, 25, 40 and 50%), sucrose (0, 1, 2, 3 and 4%), and 5 pH levels (4.5, 5, 5.5, 6 and 6.5) were tested.

The cultures were placed under continuous illumination from G.E. white fluorescent lamps (Power Groove) at 200 foot candles and at $26\pm3^{\circ}C$. For agitation of liquid medium, 50 ml Erlenmeyer flask containing 20 ml of medium was continuously agitated at approximately 160 rpm using a New Brunswick Model V shaker.

Morphological observations were made with a Wild M-5 stereomicroscope and photographed with a Nikon camera with a Micro Nikkor lens.

For anatomical study tissues were killed and fixed in Craf or FAA fixative, dehydrated in a graded series of tertiary butyl alcohol (Johansen, 1940) and embedded in paraffin. Sections 10 micron thick were stained by the rapid safranin and fast green technique (Shapiro, 1947). Observations and photographs were made with a Zeiss photomicroscope.

TABLE I

ACCESSION NUMBERS AND NAME OF PLANTS USED

Plant	Name
1183	Phal. lueddemanniana 'Fennells'[a]
1184	Phal. lueddemanniana 'Jones'
1185	Phal. lueddemanniana 'Plant A'
1186	Phal. lueddemanniana 'Div. 1'
1192	Phal. lueddemanniana 'Ochracea'
1193	Phal. cornu-cervi
1196	Phal. lueddemanniana x Phal. stuartiana
1206	Phal. lueddemanniana 'Pulchra'
1208	Phal. Luzon 'Crestwood'
1210	Phal. equestris 'New Type'
1211	Phal. equestris 'I locus del Norte'
1213	Phal. Star of Santa Cruz
1215	Phal. intermedia
1221	Phal. Dr. George Mc. Donell
1230	Phal. amabilis
1237	Dtn. Red Coral[b]
1243	Dtn. Dorette
1245	Phal. Clara Knight
1253	Phal. Pink Chiffon
1255	Phal. Susan Merkel
1302	Phal. Chieftain
1367	Phal. Golden Chief

TABLE I. (Continued) ACCESSION NUMBERS AND NAME OF PLANTS USED

Plant	Name
1380	Phal. Chieftain x Phal. Susan Merkel
1419	Phal. Clara Knight
1603	Phal. Roselle
1613	Phal. Purple Green
1618	Phal. intermedia x Phal. equestris 'I locus del Norte'
1619	Phal. intermedia x Phal. sanderana
1622	Phal. Chieftain x Phal. Zada
1624	Phal. Moonglow x Phal. Joanna Magale
1627	Phal. Doris x Phal. Susan Merkel
1669[c]	D. pulcherrima (2N)
1686	Phal. equestris x Phal. lueddemanniana
1709	Phal. Blush of Spring
1747	Phal. lueddemanniana
1748	Phal. stuartiana nobilis
1766	Phal. Bananaquit
1785	Phal. lueddemanniana x Phal. speciosa 'Orchidglade'
1793	Dtn. Coral Sand x Phal. Zada
1859	Phal. Doris x Phal. Wilder
1884	Phal. Nuel Songer x (Phal. schillerana x Phal. Sunrise)
1914	Phal. amabilis x Phal. lueddemanniana
1916	Phal. Arcadia x Phal. cochleris
1922[d]	Rnthps. Golden Dew Drops x Phal. Cassandra

TABLE I. (Continued) ACCESSION NUMBERS AND NAME OF PLANTS USED

Plant	Name
1934	D. pulcherrima (3N)
1962	Phal. equestris
1996	Phal. Zada x D. pulcherrima
1999	Phal. cornu-cervi x Phal. lueddemanniana 'Ochracea'
2156	Phal. Queen Emma
2197	Phal. lueddemanniana
2222	Phal. Terri Cook
2223	Phal. Juanita
2226	Phal. Lucifer
2388[e]	Phal. Surfrider x (Phal. Juanita x Phal. Doreen)

a Phalaenopsis
b Doritis
c Doritaenopsis
d Renanthopsis
e donated by Kodama Nursery

RESULTS

The results of clonal propagation of Phalaenopsis will be presented in two sections: in vivo experiments and in vitro experiments.

The in vivo section includes observations of spontaneous plantlet formation as well as results after induction by top-cutting or application of N-6-benzyl adenine (BA). The in vitro section includes results from aseptic culture of shoot tips, nodes of young and mature inflorescences, organ culture (leaf, stem, root) as well as investigations on culture media. Anatomical studies on growth of protocorm-like bodies (plbs) were included to help interpret morphological changes in culture.

IN VIVO EXPERIMENTS

Spontaneous

Observations of a large number of plants in the Horticulture Department and hobbyist collections showed that some Phalaenopsis species and interspecific hybrids, as shown on Table II, spontaneously produced plantlets at the node or tip of inflorescence after flowering, or on roots.

In Phal. amabilis, Phal. intermedia and Phal. equestris (Fig. 1) plantlets developed at the tip of the inflorescence after flowering. In other species, Phal. cornu-cervi and Phal. lueddemanniana (Fig. 2) plantlets formed on the nodes of the inflorescence. In the interspecific hybrid, Phal. amabilis x Phal. lueddemanniana plantlets formed on the nodes as well as on the tip of the inflorescence. In addition, a plant of Phal. stuartiana with an extensive root system on the surface of a plumeria tree in the garden of an orchid hobbyist had plantlets arising from the root at a number of places (Fig. 3).

TABLE II

SITES OF SPONTANEOUS PLANTLET PRODUCTION

Plant	Site of plantlet		
	Inflorescence		Root
	Tip	Node	
Phal. amabilis (1230)	+		
Phal. cornu-cervi (1193)		+	
Phal. equestris (1210, 1211, 1962)	+		
Phal. intermedia (1215)	+		
Phal. lueddemanniana (1206, 1183, 1184, 1185, 1186, 1192, 1747, 2197)		+	
Phal. stuartiana			+
Phal. amabilis x Phal. lueddemanniana (1914)	+	+	
Phal. cornu-cervi x Phal. lueddemanniana (1999)		+	
Phal. equestris x Phal. lueddemanniana (1686)	+		
Phal. intermedia x Phal. sanderana (1619)		+	
Phal. lueddemanniana x Phal. speciosa (1785)		+	
Phal. lueddemanniana x Phal. stuartiana (1196)		+	

Spontaneous production of plantlets

Figure 1. At tips of inflorescences after flowering in Phal. equestris (1962). 0.2x.

Figure 2. On the node of inflorescence of Phal. lueddemanniana (2197). 0.2x.

Figure 3. On the root of Phal. stuartiana. 0.2x.

Induction of Plantlets by topping

Figure 4. Two shoots formed on the basal stem of Phal. amabilis (1230) 4 months after topping. 0.4x.


1


2


3


4

Topping

When terminal cuttings of plants with a visible length of stem axis of 1230, 1245, 1302, 1380, 1419, 1748, 1793, 1916 and 1966 (2) were removed (topping), these cuttings were readily established as plants within a few weeks. After four months two shoots were produced on the remaining basal stem of 1230 (Fig. 4) and one plantlet on the other nine.

Application of N-6-benzyl adenine (BA) to inflorescences

Determination of concentration

In some Phalaenopsis species and interspecific hybrids, plantlets are formed spontaneously at the nodes or at the tips of inflorescence. This phenomenon rarely occurs in Phalaenopsis hybrids. Buds found on inflorescence node remain dormant and do not naturally form plantlets. This investigation was carried out to see if BA could be used to break bud dormancy and induce plantlet formation. The average shoot lengths 2 months after application of 0, 500, 1000 and 2000 ppm BA in lanolin paste to the two lowermost nodes of inflorescences of 1669 without bracts are shown in Table III. BA at 500, 1000 and 2000 ppm broke bud dormancy, whereas the control buds remained dormant. Since application of BA at 1000 and 2000 ppm were more effective than at 500 ppm, application of 500 ppm was omitted in the subsequent experiments.

Determination of penetration

This experiment was conducted to determine if BA would be effective when applied to nodes without removal of bract. The result obtained one month after application of 2000 ppm of BA in lanolin paste to the lowermost node of 20 inflorescences of 1215 with and without bracts is shown in Fig. 5. When 2000 ppm BA was applied to the bud with bracts

(Fig. 5 Br+) the bud showed no growth but when the bract was removed prior to application growth was observed (Fig. 5 Br-).

Determination of translocation

This experiment was carried out to determine whether BA would be translocated acropetally. The result after one month of application of 2000 ppm BA to the lowermost node of 10 inflorescences of 1934 after removing the bract is shown in Figure 6. The bud which was treated with 2000 ppm BA grew, whereas the untreated acropetal buds showed no growth.

Effect on tip and nodal bud of inflorescences

This experiment was carried out to induce plantlet formation at the tips of inflorescences. The result of 1, 2 and 6 months after 10 applications of each 0, 1000 and 2000 ppm of BA to the tip of inflorescences on 1914 are shown in Figures 7, 8, 16 and Table IV.

The untreated tips elongated only 1.8 cm in 6 months, whereas the tip elongated to an average of 10.2 cm at 1000 ppm BA and 17.3 cm at 2000 ppm BA (Fig. 16).

When BA was applied to the tip of an inflorescence, the control remained dormant after a period of 2 months, while more flowering resulted in BA treatments of 1000 and 2000 ppm (Fig. 7). After 6 months the untreated tips still remained dormant whereas at 1000 and 2000 ppm BA the tips elongated, and either plantlets formed at the tip or more flowering resulted (Fig. 8). At the point of BA application, the inflorescence axis curved and became swollen (arrow). In some instances abnormal flowers resulted on treated tips, e.g., flower without lip (arrow).

In the control, two tips produced more flowering and none formed plantlets; while at 1000 ppm two produced more flowering and five formed

plantlets; and at 2000 ppm four produced more flowering and on three tips plantlets were formed (Table IV).

The results of induction of plantlets on nodal bud of inflorescence of 1914 by BA are shown on Figures 9, 10, 11, 12 and Table V.

The effect of BA of 0, 1000 and 2000 ppm on multiple shoot formation is shown on Figure 9. After 2 months the buds in the control remained dormant, whereas one or multiple shoots formed on the 1000 and 2000 ppm BA treated nodes. After 6 months, the multiple shoots became dormant; not much growth was observed after 2 months (Fig. 10).

Table V shows that application of BA was successful in breaking dormancy of all nodal buds while most of the control buds remained dormant. Also, multiple shoot formation was obtained only on those nodes treated with BA. Few plantlets were obtained from multiple shoot formation. After an initial stimulation, most of the shoots fail to proceed any further in growth.

In addition BA at concentration of 2000 ppm was applied to the buds after removal of bracts of other hybrids of Phalaenopsis. In 8 months, buds of 2222 produced plantlets at the lowermost nodes and inflorescences at the top nodes as shown in Figure 13. Buds of 2226 (Fig. 14) produced multiple shoots at a single node, while with 1243 (Fig. 15) more inflorescences were produced in 3 months.

TABLE III

SHOOT LENGTH OF 1669 TWO MONTHS AFTER APPLICATION OF BA

BA (ppm)	No. of shoots treated	Ave. shoot length (cm)
0	20	0.1
500	20	0.3
1000	20	0.7
2000	20	0.7

TABLE IV

EFFECT OF BA ON INFLORESCENCE TIP OF 1914 AFTER SIX MONTHS

BA (ppm)	No. of tips treated	No. of dormant buds	No. of inflorescences	No. of plantlets
0	10	8	2	0
1000	10	3	2	5
2000	10	3	4	3

TABLE V

INDUCTION OF SHOOTS ON THE NODE OF INFLORESCENCE OF 1914[a] SIX MONTHS AFTER TREATMENT WITH BA

BA (ppm)	No. of nodes treated	Dormant buds	No. of nodes with			
			one shoot	one plantlet	multiple shoots	multiple shoots & one plantlet
0	20	18	1	1	0	0
1000	20	0	7	0	11	2
2000	20	0	4	0	12	4

1914[a] is an interspecific hybrid of Phal. amabilis x Phal. lueddemanniana which spontaneously forms plantlets.

Effect of BA on inflorescences in vivo

Figure 5. One month after application on buds of 1215. Growth occurred only on bud without bract (-Br). 1x.

Figure 6. One month after application on lowermost buds of 1934. Only treated buds enlarged, other buds were unaffected. 0.3x.

Figure 7. Two months after application on tip of 1914, inflorescence elongated and continued to flower. 0.3x.

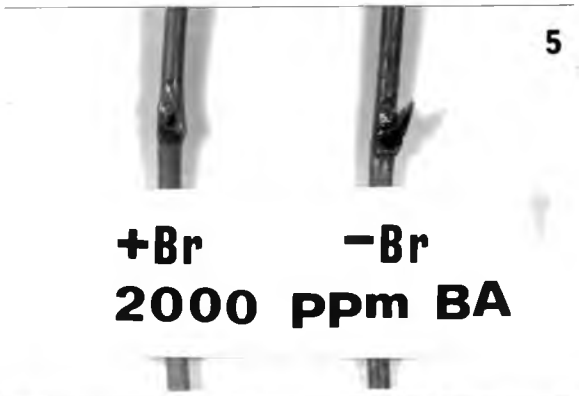
Figure 8. Six months after application on tip of 1914, inflorescence elongated and either continued to flower or produced plantlets. Horizontal line marks point of BA application. 0.1x.

Figure 9. Two months after application on nodal bud of 1914 showing single or multiple shoots. 0.6x.

Figure 10. Six months after application on nodal bud of 1914 showing single or multiple shoots. 0.6x.

Figure 11. Variations in growth of 1914 six months after application of 1000 ppm BA. 0.6x.

Figure 12. Variations in growth of 1914 six months after application of 2000 ppm BA. 0.6x.



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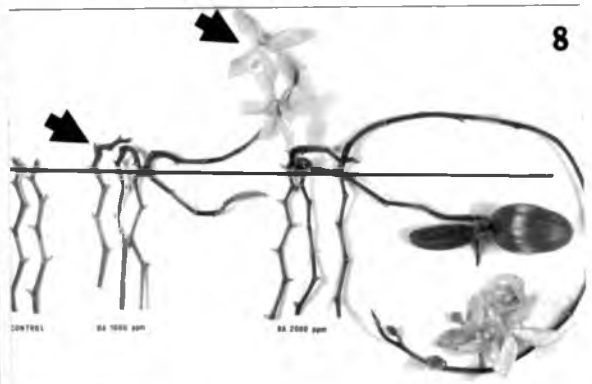

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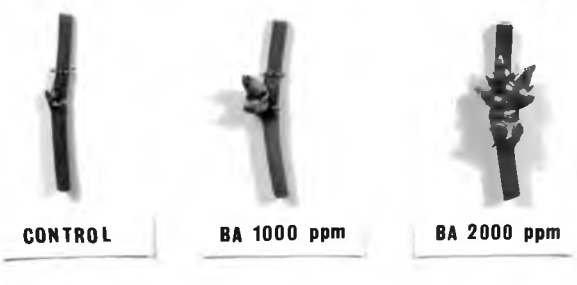

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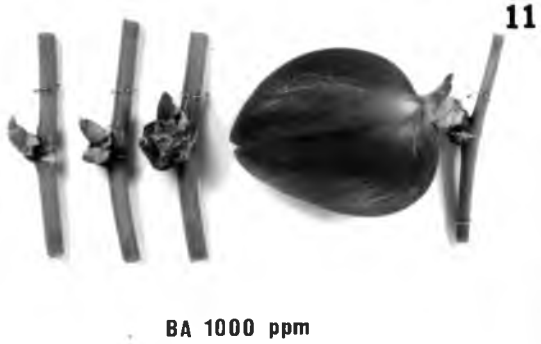

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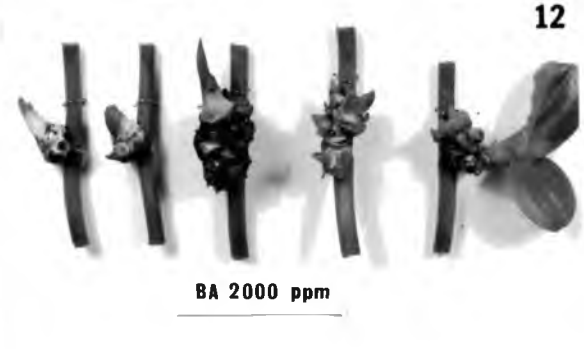

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Effect of BA in vivo

Figure 13. Eight months after application on nodal buds (arrows) of 2222, two single plantlets were produced at lowermost nodes and inflorescences at upper nodes. 0.1x.

Figure 14. Multiple shoots produced (arrow) at the lowermost node of 2226, 8 months after BA application. 0.3x.

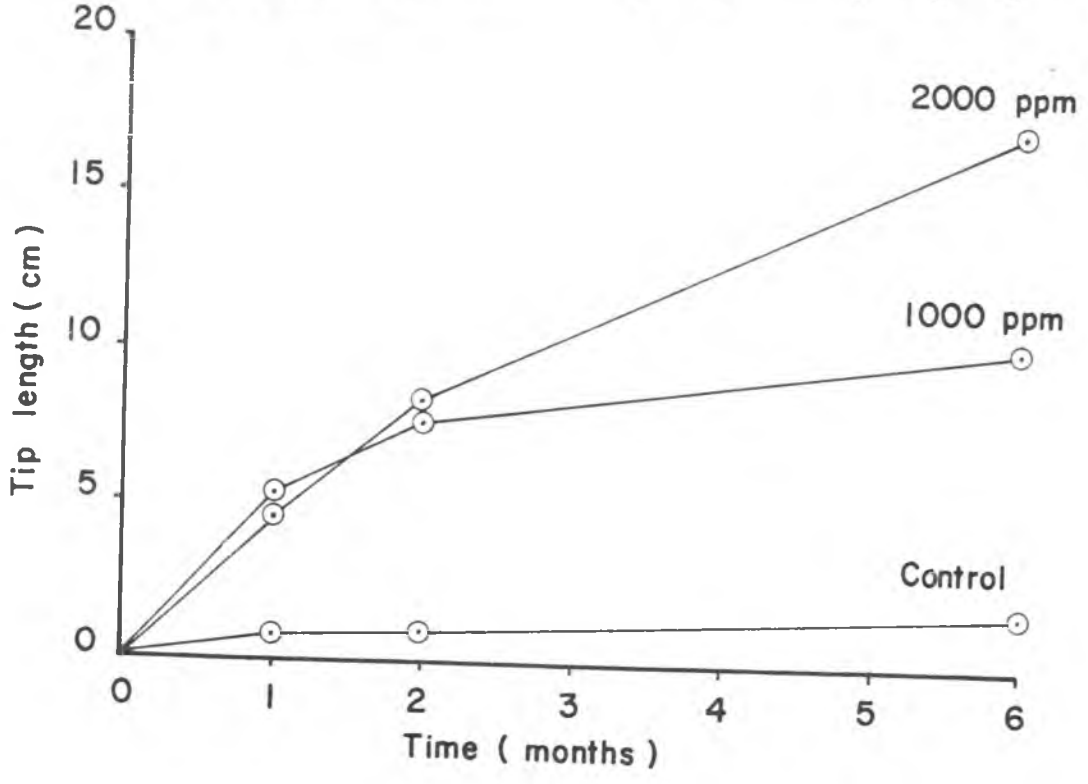
Figure 15. Production of inflorescences (arrow) at the lowermost nodes of 1243, three months after BA application. 0.1x.

Figure 16. Effect of BA on growth of inflorescence tip of 1914.

13

14

15


20
15
10
5
0
Tip length (cm)
2000 ppm
1000 ppm
Control
0
1
2
3
4
5
6
Time (months)

16

IN VITRO EXPERIMENTS

Shoot tips

When terminal and axillary buds (Fig. 17) from 10 plants of 2388 were excised and cultured in agitated liquid media for a month and transferred to solid basal media, plbs were produced within a month (Fig. 18). Since a black substance of unknown nature was elaborated from the explant into the medium, liquid medium was changed every ten days and solid medium once a month. At first plbs were yellowish in color but upon transfer to basal media minus sucrose (BM - Su) the plbs turned green and formed plantlets. Frequent subculturings resulted in multiplication of plbs to indefinite numbers.

As shown in Table VI out of 26 explants from 10 plants, 16 (61.5%) were contaminated. Of the 10 uncontaminated cultures, three (2388-1, 2388-3, 2388-4) produced plbs and one (2388-9) gave rise to a shoot, which upon re-excision gave rise to plbs.

N-6-benzyl adenine treated buds

When BA in lanolin paste at 2000 ppm was applied before excision to the 10 dormant buds of 1934, active growth occurred within one month. When these active buds (Fig. 19) were excised, sterilized and cultured in agitated liquid basal media for a period of a month, seven explants produced plbs in another month upon transferring to solid basal medium (Fig. 20). When subcultured to solid BM - Su multiplication occurred.

Inflorescence

Mature inflorescence node propagation

The results obtained from culturing mature inflorescence nodal

sections from 20 hybrids on solid basal media (BM) for 2 months are shown in Table VII

Seventy five explants out of 392 (19.1%) were contaminated. Of 317 (80.9%) uncontaminated cultures, 124 (39.1%) showed no growth, 11 (3.5%) formed plbs (Fig. 21), 151 (47.6%) formed one to four plantlets per node (Fig. 23), and 31 (9.8%) formed inflorescences (Fig. 24) in two months which later produced plantlets at the tip or at the lowermost node of the emerging inflorescence.

In three months plantlets were well rooted and ready for potting in the greenhouse. In the case of 1213, 1230 and 1267 when the inflorescence nodal section with plantlet from which leaves had been excised were cultured in agitated liquid BM for one month and transferred to solid medium, plbs were obtained in another month (Fig. 25). The rest produced plantlets from axillary bud (Fig. 26).

Mature inflorescence node propagation from different positions

The results after 2 months from culturing a total of 80 inflorescence nodal sections from four positions numbered acropetally on solid BM medium are shown in Table VIII.

None of the buds from position 1 formed inflorescences whereas 13.3%, 33.3% and 86.7% of cultures from positions 2, 3 and 4 respectively, produced inflorescences. More plantlets were produced at the lower nodes, 50.0%, 60.0%, 33.3% from positions 1, 2 and 3 respectively. Only 1 (6.7%) out of 20 produced plantlets from position 4. Plbs were formed by buds from positions 1 to 3.

Figures 33, 34, 35, 36 show the variation in type of growth at each nodal position after 4 months. The higher the node the tendency

to form inflorescences was greater. The number of plantlets produced at the tip or from the lowermost node of the emerging inflorescence was less than that formed directly from node.

Mature inflorescence node and bud propagation on basal medium (BM)

The results of 20 inflorescence nodes and 20 inflorescence buds of 1785 grown on basal medium for 2 months are shown in Figure 27. All of the inflorescence nodal cultures formed plantlets. Only 6 out of 20 inflorescence buds produced plantlets; the rest showed no growth (Fig. 29). Paraffin sections revealed meristematic areas as shown in Figure 28 forming two shoots. In the inflorescence bud culture (Fig. 29), the cells at the tip were larger and vacuolated and not actively dividing (Fig. 30).

Mature inflorescence node and bud propagation on BA medium (BM + BA)

Phalaenopsis which cannot grow on basal media

The results of five inflorescence nodes of 1622 and 1916 each grown in various concentrations of BA (0, 1, 5, 10 ppm) are shown in Figures 31, 32. After 2 months it was found that 5 ppm BA caused rapid shoot formation in 1622 (Fig. 31), and multiple shoot formation in 1916 (Fig. 32).

Phalaenopsis which can grow on basal media

The results of five inflorescence nodes and buds of 1785 grown in various concentrations of BA media (0, 1, 5, 10, 20 ppm) are shown in Figures 37, 38. After 4 months, plantlets were formed in basal media. All cultures grown in BA had yellow leaves, while cultures grown in 5 ppm or more BA had elongated or multiple shoots without roots.

With inflorescence buds (Fig. 38) in basal medium there was no growth, with 20 ppm BA the buds died. BA at 1 ppm promoted rapid shoot formation and at 5 and 10 ppm multiple shoot formation.

Mature inflorescence node and bud propagation on NAA medium (BM + NAA)

The results of application of varying amounts of NAA (0, 1, 5, 10 ppm) on inflorescence node sections of 1785 after 4 months are shown in Figure 39. There were more roots in NAA treated node sections than in control. Shoot growth was inhibited in NAA medium.

When inflorescence bud of 1785 was grown in BM (Fig. 40), no growth was observed. With addition of NAA shoot growth was inhibited but root formation was stimulated.

Young inflorescence node and tip propagation on 5 ppm BA medium

The results after 2 months in culture of inflorescence nodes and tips of 15 clones (1302, 1380, 1622, 1624, 1627, 1709, 1748, 1766, 1793, 1859, 1884, 1916, 1922, 2013, 2223) in relation to their position on the inflorescence are shown in Table IX. The percentage of dormant or dead buds decreased from the tip of the inflorescence to the lower nodes. The number of plantlets produced increased besipetally. If a bud produced an inflorescence, more buds appeared later at the lowermost node (Fig. 41) on the newly formed inflorescence of 1793.

Growth of shoot shown in Figure 42 for 1748 was best from positions 3 and 2.

TABLE VI

SHOOT TIP CULTURES AFTER TWO MONTHS

Plant	Explants	Contaminated	Uncontaminated	Dead	Explants producing	
					Plbs	Plantlets
2328-1	4	2	2	1	1	0
2328-2	3	2	1	1	0	0
2328-3	3	1	2	1	1	0
2328-4	4	2	2	1	1	0
2328-5	2	1	1	1	0	0
2328-8	2	2	0	0	0	0
2328-9	2	1	1	0	0	1
2328-11	2	2	0	0	0	0
2328-12	2	2	0	0	0	0
2328-18	2	1	1	1	0	0
Total	26	16	10	6	3	1
Percentage	100	61.5	38.5	60.0[a]	30.0[a]	10.0[a]

a Percentage calculated from uncontaminated.

TABLE VII

MATURE INFLORESCENCE NODE PROPAGATION AFTER TWO MONTHS

Plant	Explants	Conta-minated	Uncontaminated	Cultures producing			
				dormant or dead buds	plbs	plantlets	inflores-cence[a]
1208	3	1	2	0	0	2	0
1213	11	2	9	5	0	3	1
1221	2	0	2	0	0	2	0
1237	7	0	7	1	1	5	0
1243	60	14	46	28	1	15	2
1245	8	2	6	6	0	0	0
1253	2	1	1	1	0	0	0
1255	13	3	10	2	0	8	0
1302	16	1	15	15	0	0	0
1367	3	0	3	3	0	0	0
1603	9	1	8	0	0	8	0
1613	27	8	19	6	0	13	0
1622	7	6	1	1	0	0	0
1624	16	1	15	6	0	9	0
1669	60	5	55	6	9	20	20
1748	3	2	1	1	0	0	0
1766	9	8	1	0	0	1	0
1914	108	16	92	30	0	54	8
1916	26	2	24	13	0	11	0
2156	2	2	0	0	0	0	0
Total	392	75	317	124	11	151	31
Percentage	100	19.1	80.9	39.1[b]	3.5[b]	47.6[b]	9.8[b]

a Plantlets formed after inflorescence.
b Percentage calculated from uncontaminated.

TABLE VIII

MATURE INFLORESCENCE NODE PROPAGATION FROM DIFFERENT POSITIONS AFTER TWO MONTHS

Explant position[a]	Cultures	Contaminated	Uncontaminated	Cultures Producing			
				dormant	plbs	plantlets	inflorescence[b]
1	20	10 (50.0)[c]	10 (50.0)	1 (10.0)[d]	4 (40.0)	5 (50.0)	0 (0.0)
2	20	5 (25.0)	15 (75.0)	3 (20.0)	1 (6.7)	9 (60.0)	2 (13.3)
3	20	5 (25.0)	15 (75.0)	1 (6.7)	4 (26.7)	5 (33.3)	5 (33.3)
4	20	5 (25.0)	15 (75.0)	1 (6.7)	0 (0.0)	1 (6.7)	13 (86.7)

a numbered acropetally.
b plantlets formed after inflorescence.
( )[c] percentage.
( )[d] percentage calculated from uncontaminated.

TABLE IX

EFFECT OF 5 PPM BA ON YOUNG INFLORESCENCE NODE AND TIP OF 15 CLONES AFTER TWO MONTHS

Explant position[a]	Cultures	Contaminated	Uncontaminated	Cultures producing			
				dormant	plbs	plantlets	inflorescence[c]
1	18	0 (0.0)[d]	18 (100.0)	4 (22.2)[e]	3 (16.7)	9 (50.0)	2 (11.1)
2	22	4 (22.2)	18 (77.8)	7 (38.9)	0 (0.0)	8 (44.4)	3 (16.7)
3	31	14 (45.2)	17 (54.8)	7 (41.2)	3 (17.6)	5 (29.4)	2 (11.8)
T[b]	31	9 (29.0)	22 (71.0)	18 (81.8)	0 (0.0)	4 (18.2)	0 (0.0)

a numbered acropetally.
b is terminal.
c plantlets formed after inflorescence.
( )[d] percentage.
( )[e] percentage calculated from uncontaminated.

Shoot tip culture of 2388

Figure 17. Shoot tip exposed by removal of leaves. Note terminal and axillary buds used as explants. 2.5x.

Figure 18. Protocorm-like bodies (plbs) formed 2 months after culturing on basal medium (BM). 2.5x.

BA treated bud cultures of 1934

Figure 19. Active buds ready for excision one month after BA treatment. 1x.

Figure 20. Plbs formed 2 months after culturing on BM. 2.5x.

Mature inflorescence node cultures after 2 months on BM

Figure 21. Plbs on 1669. 2x.

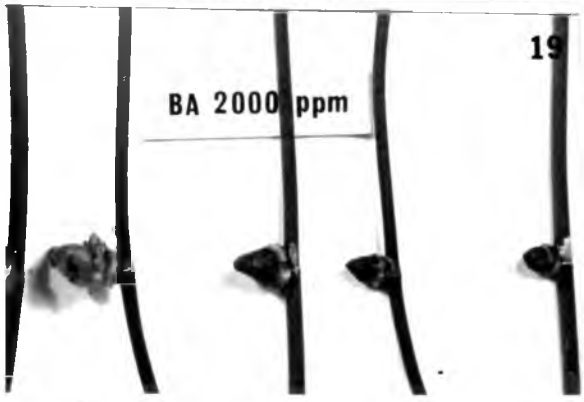
Figure 22. Plbs and plantlets on 1367. 2.5x.

Figure 23. Plantlet on 1213. 2x.

Figure 24. Inflorescence on 1669. 2x.


17


18


19
BA 2000 ppm


20


21


22


23


24

Mature inflorescence node and bud cultures in vitro

Figure 25. Inflorescence nodal culture of 1213 in BM 2 months after removal of leaves on plantlet. Note the formation of plbs. 2.5x.

Figure 26. Inflorescence nodal culture of 1213 in BM 2 months after removal of leaves on plantlet. Note the formation of shoots. 5x.

Figure 27. Growth on inflorescence node and no growth of bud of 1785 after 2 months. 0.5x.

Figure 28. Longitudinal section of inflorescence node showing 2 adventitious buds. 40x.

Figure 29. Bud showing no growth after 2 months on BM. 4x.

Figure 30. Longitudinal section of bud showing shoot apex with no dividing cells. 200x.

Figure 31. Two months after culture of 1622 in BA. Shoot grew best in 5 ppm BA. 0.5x.

Figure 32. Two months after culture of 1916 in BA. Multiple shoots were produced in 5 ppm BA. 0.5x.

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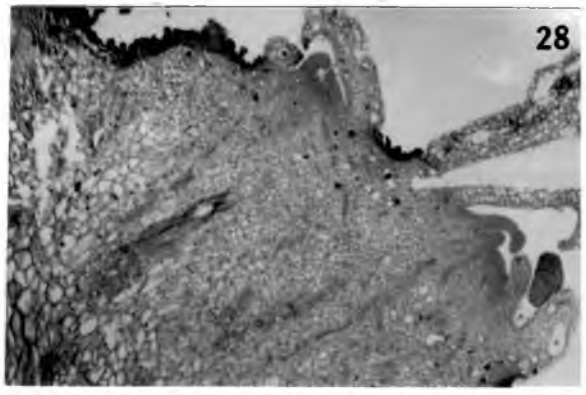

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32

Mature inflorescence node cultures of 1669 after 4 months on BM

Figure 33. Position 1 showing dormant bud, plb and plantlet formation. 0.5x.

Figure 34. Position 2 showing plb, plantlet and inflorescence formation. 0.5x.

Figure 35. Position 3 showing plb, plantlet and inflorescence formation. 0.5x.

Figure 36. Position 4 showing inflorescence formation and plantlets formed at the tip or at the node of emerged inflorescence. 0.5x.

Mature inflorescence node and bud cultures of 1785 after 4 months

Figure 37. Inflorescence node cultures on BA media showing the number of shoots increased with concentration but no roots are formed. 0.5x.

Figure 38. Inflorescence bud cultures on BA media showing buds died at 20 ppm concentration. Multiple shoots formed on 5-10 ppm BA. 0.5x.

Figure 39. Inflorescence node cultures on NAA media showing two roots formed on 5-10 ppm NAA. Shoot length decreased with increased concentration. 0.5x.

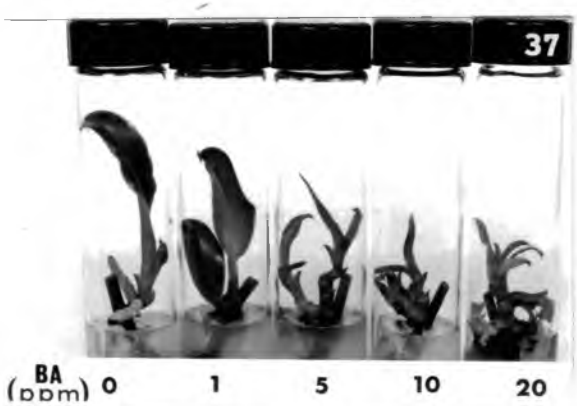
Figure 40. Inflorescence bud cultures on NAA media showing number of roots increased with concentration. 0.5x.


33
1


34
2


35
3


36
4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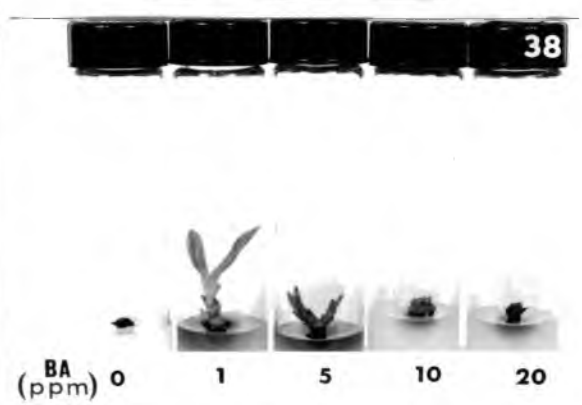
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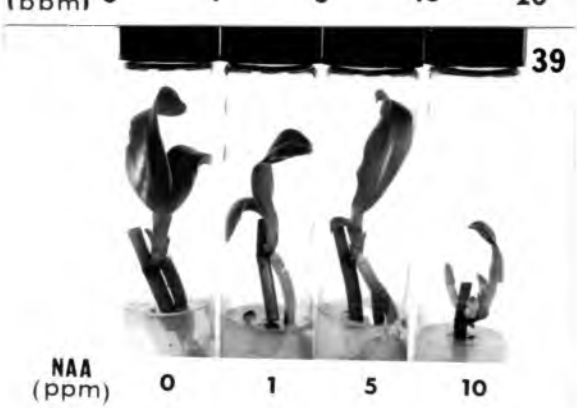
BA (ppm) 0 1 5 10 20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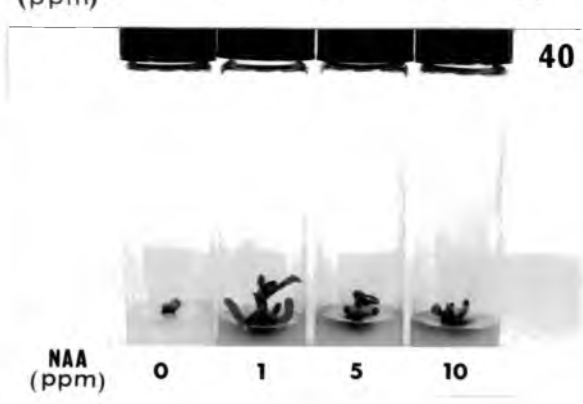
NAA (ppm) 0 1 5 10


40
NAA (ppm) 0 1 5 10

Young inflorescence node cultures on 5 ppm BA after 2 months

Figure 41. Shoot developed from cultures of lowest node (1) of 1793. In cultures of nodes 2 and 3 inflorescences emerged which, in turn, produced plantlets on the proximal node of the newly emerged inflorescence. The tip (T) died. 0.5x.

Figure 42. No growth occurred in culture of node 1 of 1748 whereas plantlets were produced on nodes 2 and 3. The tip (T) died. 0.5x.

Leaf cultures of 1237 in liquid VW + 50%CW - Su

Figure 43. Two month old culture showing root and leaf-like appendages on leaf sheath. 6x.

Figure 44. The same leaf culture as Figure 43 after 3 months showing plantlets formed from leaf-like appendages. 5x.

Figure 45. Two month old culture showing adventitious buds from leaf base. 6x.

Figure 46. Longitudinal section of leaf showing 2 adventitious buds forming from meristem on leaf sheath. 40x.

Cultures of Leaf, stem, and root from Plantlet of 1914 on BM

Figure 47. Stem produced 5 shoots in one month, root elongated, but leaves showed no further growth. 1x.

Figure 48. The same culture as in Figure 47 a month later. Roots continued to elongate, shoots developed roots, and some leaves died. 1x.

41

1 2 3 T

42

1 2 3 T

43

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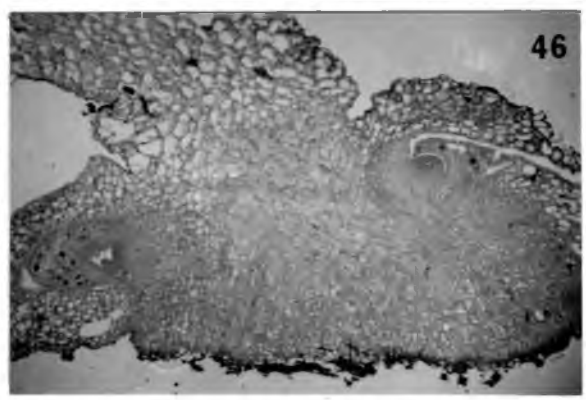

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Tumor growth

Figure 49. Excised stem of 1213 grown on BM + 1 ppm 2,4-D for 3 months showing tumor growth. 8x.

Figure 50. Longitudinal section of tumor growth shown in Figure 49. Epidermis, cortex and vascular cylinder are present in the protuberance. 20x.

Figure 51. Plantlet of 1213 grown on BM + 1 ppm 2,4-D for 2 months showing tumor at the base of stem and tip of root. 3x.

Figure 52. Longitudinal section of tumor at the base of the stem of plantlet in Figure 51 showing protuberance of tissues from the endogenous tissues of stem. 40x.

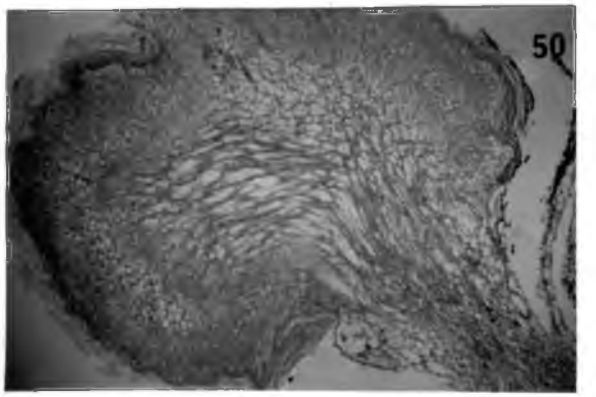
Figure 53. Excised root of 1213 grown on BM + 1 ppm 2,4-D for 3 months showing tumors on the root. 3x.

Figure 54. Longitudinal section of tumor at the tip of the root showing its endogenous origin. 40x.

Figure 55. Longitudinal section of 3 month old tumor along the root shown in Figure 53. Tumor has two broad meristematic zones. 30x.

Figure 56. Longitudinal section of a normal root tip showing root cap (RC), initial cells (I), epidermis (E), cortex (C), and vascular cylinder (V). 40x.


49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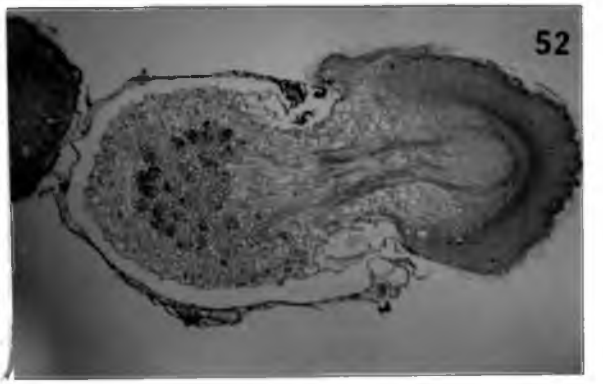
51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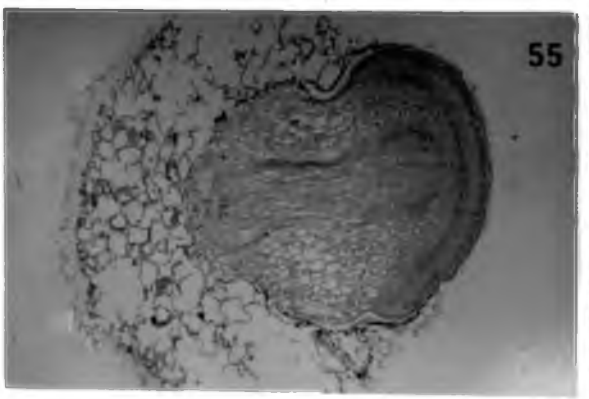

55


56
E
C
V
RC
C

Tumor growth and plantlet formation

Figure 57. Cross section of 3 month old tumor shown in Figure 55, showing outer meristematic zone, root cap (RC), epidermis (E), cortex (C) and vascular cylinder (V). 30x.

Figure 58. Cross section of 3 month old tumor shown in Figure 55, showing the outer and inner meristematic zones. 30x.

Figure 59. Cross section of 5 month old tumor. Cells of outer root cap have degenerated. The meristematic zone is still clearly visible. 40x.

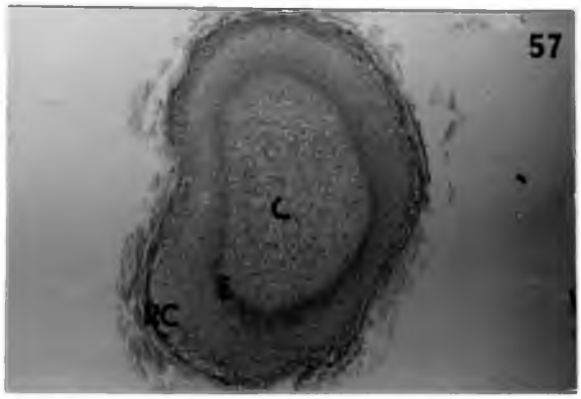
Figure 60. Enlargement of meristematic zone in Figure 59 showing outward production of cells. 100x.

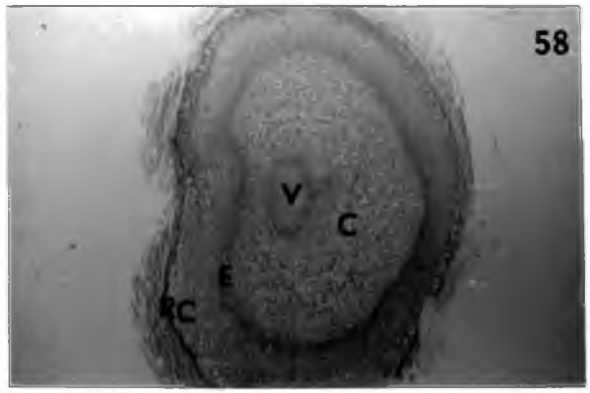
Figure 61. Tumor from root on BM - Su after 5 months. 3x.

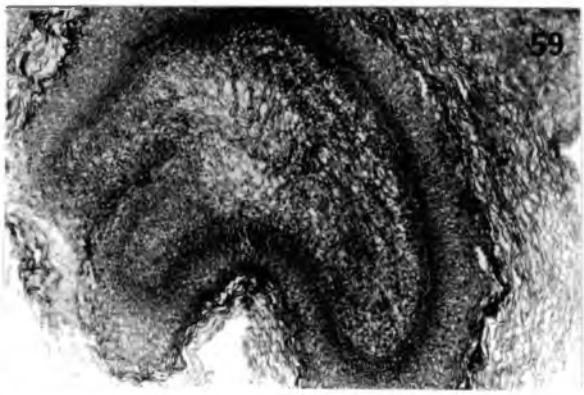
Figure 62. Tumors cultured for 9 months on 2,4-D containing medium (left) and on medium without 2,4-D and sucrose (right). In flask on right plbs and plantlets are present. 0.5x.

Figure 63. Cultures of tumors from 1213 (left), 1230 (center) and 1267 (right) on BM - Su after 9 months showing plantlets. 0.3x.

Figure 64. Close up of flask on right in Figure 62 showing tumor, plbs and plantlets. 2x.


57
C
E
RC


58
V
C
E
RC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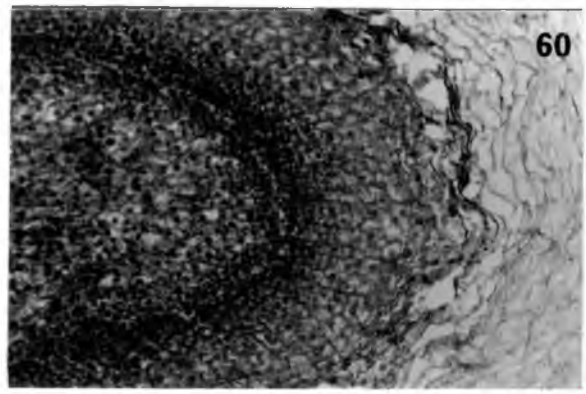
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61


62


63


64
No. 49
STOPPER

TABLE X

PLANTLETS AND TUMORS FROM ORGAN CULTURE OF THREE CLONES AFTER THREE MONTHS

Media	Leaf		Stem		Root	
	Plantlets	Tumor	Plantlets	Tumor	Plantlets	Tumor
BM (VW+15%CW+Su)	-[a]	-	+[b]	-	-	-
VW + 50%CW - Su	+	-	++	-	-	-
BM + 1 ppm 2,4-D	-	-	-	+++	-	++

-[a] no response
+[b] response

Organ

Leaves, stems and roots were excised from aseptically grown plantlets at the three leaf- and two root-stage and cultured in three kinds of media: VW + 15%CW + Su (BM), VW + 50%CW - Su, and BM + 1 ppm 2,4-D. A total of fifteen leaves, five stems, and ten roots of each mericlone of 1213, 1230 and 1267 were used. The results are shown in Table X.

Plantlets were obtained from stems cultured on BM and VW + 50%CW - Su, and leaves cultured on VW + 50%CW - Su. Tumor growth was induced in stem and root cultures grown on 2,4-D medium.

Leaf culture

When leaves from plantlets of 1237, 1916 and 1914 were excised and grown in agitated VW + 50%CW - Su for one month and then kept in stationary liquid medium, a black substance diffused from the leaf necessitating the change of medium every 10 days. Shown in Figure 43 is the leaf culture of 1237. One root appeared 40 days after excision near the cut end, and grew toward the abaxial side. Leaf-like appendages appeared on the adaxial side along the cut end. About 2 weeks after the formation of the appendages, a number of buds were formed along the base of the appendages. A month later, roots appeared on the buds as shown in Figure 44.

In other cultures, leaf-like appendages did not appear but buds formed in about 2 months at the cut end of the leaf (Fig. 45).

A longitudinal section of the leaf showed that these adventitious buds arose endogenously from the basal meristem (Fig. 46). The buds have a narrow dome-shaped shoot apex covered by the first leaf and are similar in structure to a typical shoot.

In these cultures, the adventitious buds did not show a protocorm-like stage prior to shoot differentiation.

The number of plantlets formed from leaf cultures varied among the hybrids. In the case of 1237 from 4 to 14 plantlets were obtained per culture, in 1916 3 or 4 plantlets, and in 1914 1 plantlet.

Stem culture

Stem cultures produced plantlets from axillary buds in media with 15% and 50% coconut water. As shown in Figure 47 five shoots were obtained in one month from a single stem of 1914 grown on 15% CW medium. One month later the plantlets rooted (Fig. 48).

2,4-D induced tumor growth on the excised stem of 1213 as shown in Figure 49, and on the whole plantlet as shown in Figure 51. The stem tumor was a protuberance with a shiny smooth surface by the second month and later became rough surfaced with masses of dead white cells. The tumor turned yellow and died if allowed to continue on the 2,4-D medium. When the tumor was removed and placed on 2,4-D free medium, protocorm-like bodies differentiated on the tumor (Fig. 62). Some of the plbs formed plantlets while others continued to proliferate and form more plbs (Figs. 63, 64).

When the intact stem was grown in 2,4-D medium, tumor growth was induced on the stem as well as on the tip of the root (Fig. 51). The stem tumor was much more active and progressively enlarged. The root tumor remained quiescent. Anatomical studies showed that tumors formed on both stems and roots originated in much the same fashion as an adventitious root (Figs. 52, 54, 55).

The origin of the tumor growth was internal with respect to the

cortex and epidermis as shown in Figure 52. The central part is the vascular cylinder, the enlarged principal conducting and strengthening portion. The outermost tissue of vascular cylinder grows outward to form the tumor. Cap and root initials differentiated but the meristematic zones were much broader than in a normal root. Cell divisions and elongation in these two meristematic zones caused the tumor to push outward through the cortex and epidermis to form round greenish bodies externally.

Root culture

Tumor growth was obtained by culturing root tips 1 cm in length on solid BM + 2,4-D (0.5, 1, 2, 3, 4 ppm). There was a positive response to 2,4-D at lower concentrations of 0.5 and 1 ppm. Higher concentrations of 2,4-D (2,3,4 ppm) resulted in death of the root. Tumor growth appeared near the tip, at the tip and in all directions from the root axis (Fig. 53). During the first month, the initially pointed and green root tip turned yellow and brown. Later, the root cap cracked and the tumor growth protruded from the root. Tumor growth along the root followed the same pattern of development as branch roots. They appeared as smooth greenish protuberances with a shiny surface that became whitish as they grew. The surface of the tumor growth became rough in the third month of culture (Fig. 53).

Anatomically, the tumors probably originated endogenously from the pericycle, a layer of parenchyma cells capable of producing new cells that grow outward and form branch roots. The tumor pushed outward through the cortex of the root and multiple epidermis. The tumor appeared to be similar in structure to a branch root, except that roots

normally grow in one direction (elongation), while the 2,4-D induced tumor growth grows in all directions due to the presence of two broad meristematic zones. A many cell layered root cap covers the tumor and progressive death of the outermost layers gives the tumor surface a rough, whitish appearance (Fig. 61).

When tumor growth was left on solid medium with 2,4-D the explanted root and tumor turned yellow and died. When the tumor is transferred to 2,4-D free medium (BM -Su) it can survive although the growth was slow. It eventually developed plbs followed by plantlets.

Media studies

The percentage increase in fresh weight of plbs and plantlets after 40 days in culture with Vacin and Went basal medium modified by adding various concentrations of coconut water (0, 15, 25, 40 and 50%), sucrose (0, 1, 2, 3 and 4%) and at different pH levels (4.5, 5, 5.5, 6 and 6.5) is shown in Figure 65. The colors of plbs and plantlets were recorded and shown in Figures 66, 67.

For plbs in media without sucrose and with 0-50% CW, the fresh weight increased to a maximum of 80% with 15%CW. With no coconut water, no increase in fresh weight was observed in 40 days.

When sucrose in medium was varied from 0-4% fresh weight decreased from 86% without sucrose to 45% with 4%.

When the pH was adjusted between 4.5 to 6.5, the increase in fresh weight varied from a high of 88% at pH 6 to a low of 74% at pH 6.5.

For plantlets grown in varying concentrations of coconut water, increase in fresh weight ranged from 50% for control to a peak of 80% with 40%CW. When sucrose ranged from 0 to 4% the increase was between

50 to 60%. When pH was between 4.5 to 6.5, the increase in fresh weight varied between 60 to 70%.

As shown in Figure 66 when plbs were grown in media with various concentrations of coconut water and sucrose for 40 days plbs were greenest in 0% sucrose. With increasing concentrations of coconut water, the degree of yellowing increased. In 40 and 50%CW many brown plbs appeared.

In media without sucrose, plb was green, whereas increasing amounts of sucrose resulted in larger numbers of brown plbs. Changes in pH did not show any effect on color of plbs.

There were no differences in color for plantlets grown in varying concentrations of coconut water, sucrose and levels of pH (Fig. 67).

Stages of plb development

Plbs of less than 0.5 mm in size were small globular structures with rough surfaces (a in Fig. 68). An anatomical study showed that they were composed of 1 to 30 meristematic cells.

When between 0.5 - 1 mm in diameter, they were globular in shape, with a shiny surface, (b in Fig. 68). Many trichomes (rhizoids) were on the surface of the lower half of plbs. These globular plbs were comprised of about 30 cells in diameter and included one layer of epidermis surrounding a ground tissue of large parenchyma cells.

When plbs are 1 - 3 mm in diameter (c in Fig. 69) a dark green spot marked the point on each plb where the first leaf later appeared (d in Fig. 69). Numerous rhizoids were present. A cross section showed a root primordium.

When the plb was approximately 4 mm in diameter the first leaf expanded and a root near the base of the leaf was present (e in Fig. 70).

As shown as in f in Figure 71 the plb remained 4 mm in diameter but the leaf enlarged and the root elongated showing an area with velamen and root tip. It took 3 months from stage a to f.

Subculture of plb

If plbs were left on solid media, each plb formed one plantlet. If plb was subcultured in new medium at the first leaf stage, plbs appeared at the base and progressively toward the tip while the first leaf continued to expand (Fig. 72). In some cultures the original plb died (arrow) and in some cultures roots were formed (arrow).

When plb was subcultured at the first leaf and first root stage, only one plantlet was produced. These plbs grew rapidly in sucrose medium. Occasional plbs were produced at the base. When root and leaf were excised from these plantlets and only the swollen base was cultured, new plbs were formed (Fig. 73).

When the stem from plantlets with four leaves and two roots was cultured in BM after excision of leaves and roots, 4 to 5 shoots were produced.

Origin of plb

Subculture of plbs at one leaf stage resulted in new plbs near the base and progressively to the tip of each plb. In some cases new plbs formed at the tip of the leaf and subsequently toward the leaf base. More plbs were formed on the upper (adaxial) than on the lower (abaxial) epidermis.

Origin of new plbs on the old plb is shown in Figure 75. A cell in the epidermis divided anticlinally forming two protruding cells. These cells were active, having large nuclei and dense cytoplasm. Further divisions of the cells in various directions produced a globular-shaped

structure with isodiametric cells with large nuclei and dense cytoplasm (Figs. 76, 77). The point of attachment between new and old plb is narrow as shown in Figure 77. The cells continued to divide in a certain area, and enlarged in the other. Figure 78 shows that enlargement of cells especially the area furthest from the point of attachment to the old plb. One well-defined layer of epidermis was observed at this stage. Along the surface of the lower parts, trichomes which are multicellular and containing dense cytoplasm and large nuclei was present (Fig. 82). At the tip of each plb, an apical meristem was formed (Fig. 79) before root initial (Fig. 80) appeared below the shoot apex. When first leaf and first root were well established, starch grains were accumulated in the cells of ground plb (Fig. 74).

Origin of new plb formed on first leaf is shown in Figure 83. A single cell on the upper epidermis of young leaf became active and eventually formed a single plb. This cell divided to form a globular structure (Figs. 84, 85), composed of meristematic cells with large nuclei and dense cytoplasm. The cells on one side of the globular structure enlarged while the cells in the other portion remained meristematic (Fig. 86). The meristematic region will form the shoot tip.

Several adjacent cells of the upper epidermis became active (Fig. 87), a cluster of plbs was eventually formed by divisions of the active epidermal cells. A flattened mass of tissue was first formed (Figs. 88, 89) and within this mass, several plbs differentiated (Fig. 90).

Figure 65. Effect of coconut water, sucrose and pH levels on growth of protocorm-like bodies and plantlets of 1213, 40 days after culture.

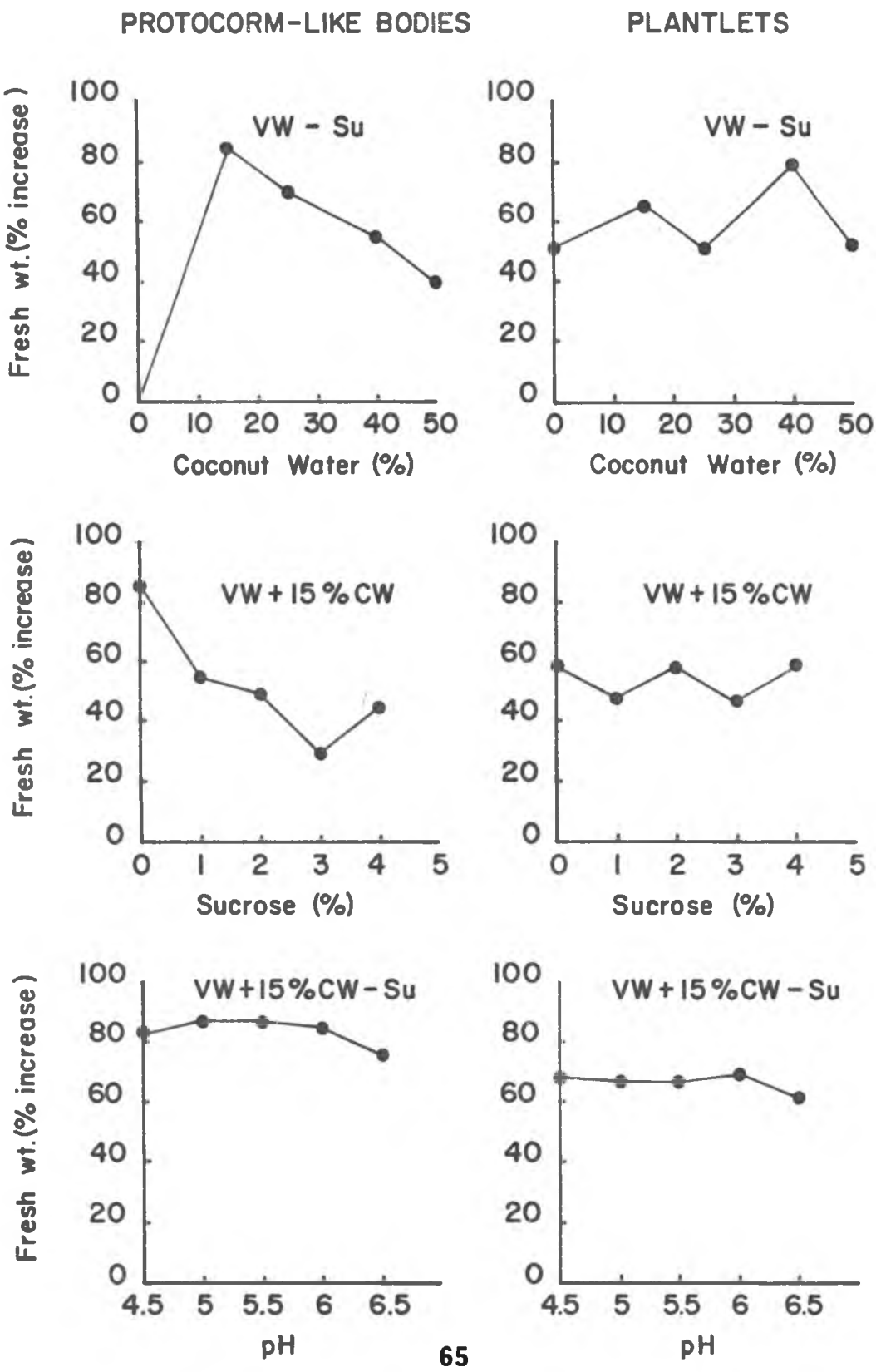
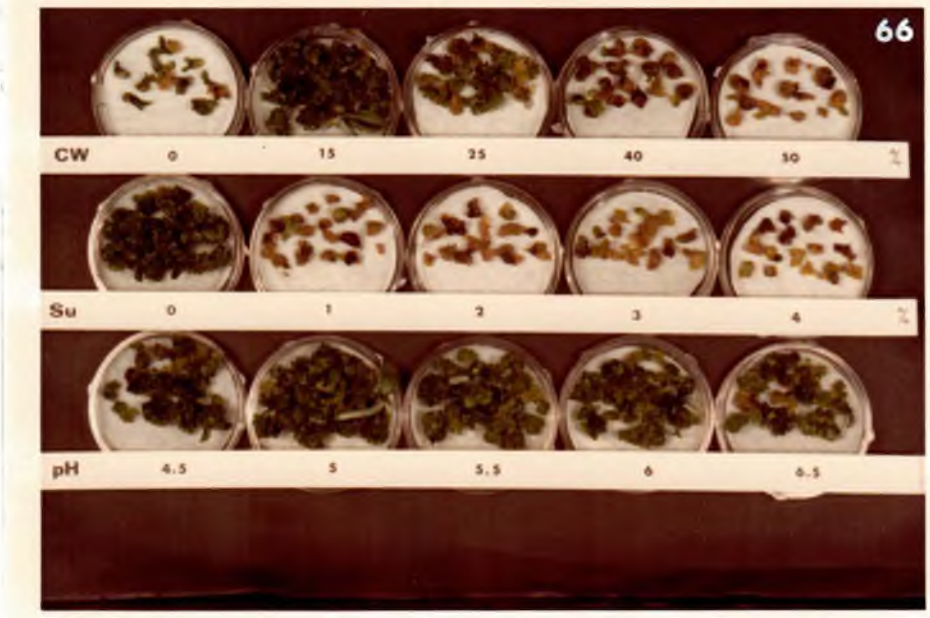
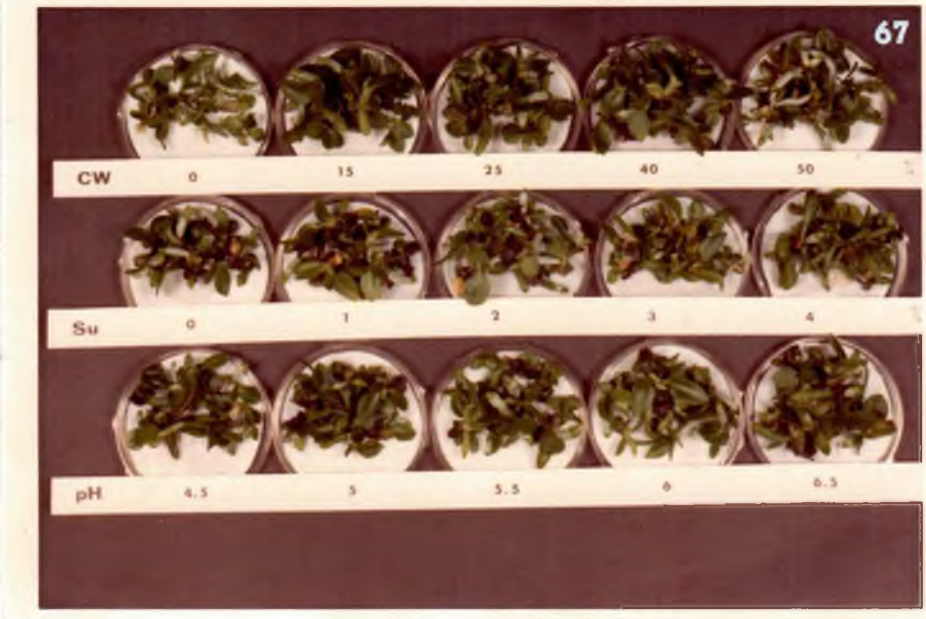

PROTOCORM-LIKE BODIES
PLANTLETS
Fresh wt.(% increase)
VW - Su
Coconut Water (%)
VW + 15 % CW
Sucrose (%)
VW + 15 % CW - Su
pH
0
20
40
60
80
100
0 10 20 30 40 50
0 1 2 3 4 5
4.5 5 5.5 6 6.5

Figure 66. Effect of various concentrations of coconut water, sucrose and pH levels on color of plbs of 1213 40 days after culture. 0.3x.

Figure 67. Effect of various concentrations of coconut water, sucrose and pH levels on color of plantlets of 1213 40 days after culture. 0.3x.


66
CW 0 15 25 40 50 %
Su 0 1 2 3 4 %
pH 4.5 5 5.5 6 6.5


67
CW 0 15 25 40 50 %
Su 0 1 2 3 4 %
pH 4.5 5 5.5 6 6.5

Stages of plb development of 1213

Figure 68. One month old culture showing small, globular plbs with rough surface, less than 0.5 mm in diameter (stage a) and plbs of 0.5-1 mm in diameter with smooth surface and trichomes (stage b). 2x.

Figure 69. Two month old culture showing plbs 1-3 mm in diameter with dark green tip (stage c) and 4 mm plbs with first leaf (stage d). 2x.

Figure 70. Three month old culture showing 4 mm plbs with first leaf and root (stage e) and fully-formed plantlets (stage f). 2x.

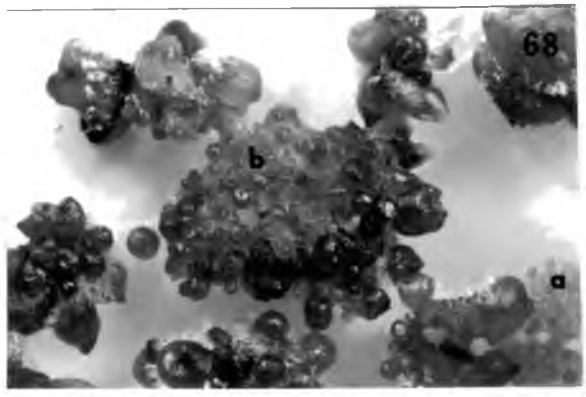
Figure 71. Another 3 month old culture showing fully formed plantlets (stage f). 2x.

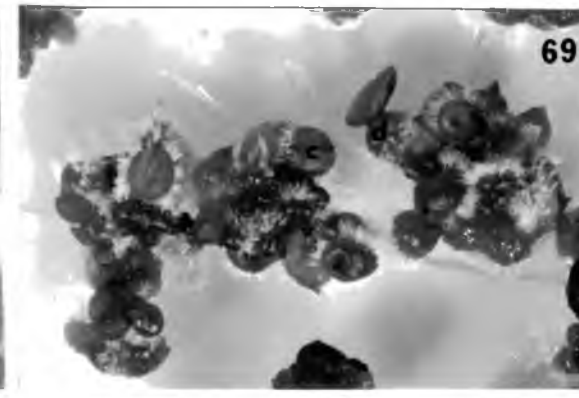
Subculture of plbs

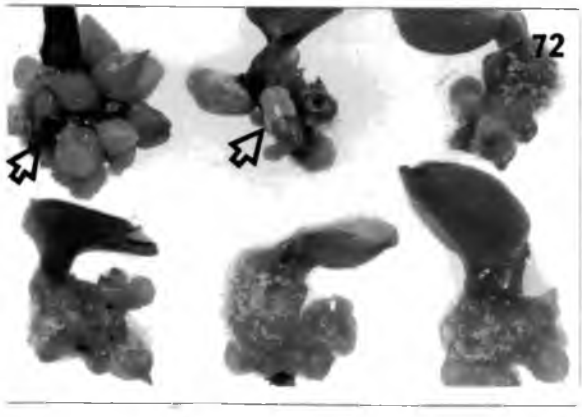
Figure 72. Young plbs produced on the original plbs after 2 months. 2x.

Figure 73. One month after excision of leaf from plb, more plbs are produced leaving a degenerated original plb. 2x.

Figure 74. Cross section of plb with single leaf and root. Shoot apex (S), root (R), and parenchymatous cells impregnated with starch grains (P). 20x.







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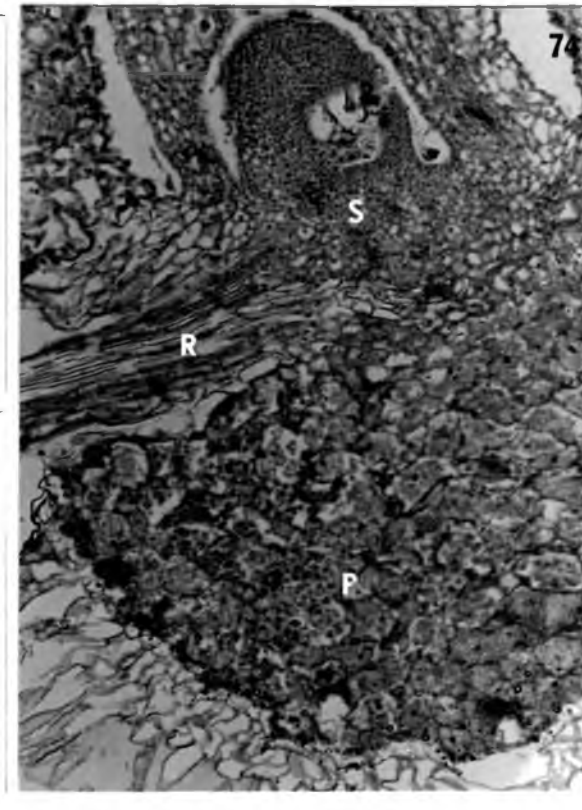


Formation and organization of plb on the epidermis of old plb

Figure 75. Longitudinal section of original plb after 1 month showing two active cells at the epidermis. 160x.

Figure 76. Longitudinal section of original plb after 1 month showing globular structure containing meristematic cells. 160x.

Figure 77. Longitudinal section of original plb after 1 month showing the connection of plbs. 160x.

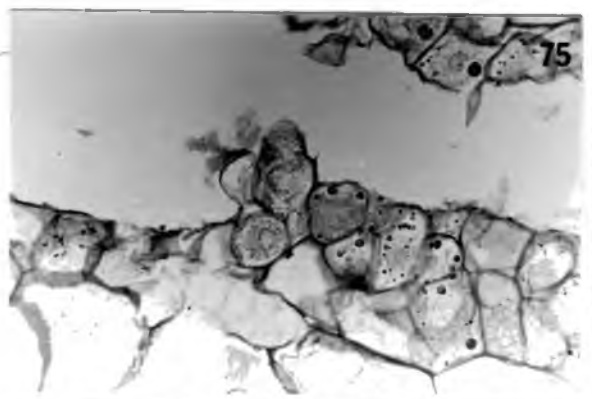
Figure 78. Longitudinal section of developing plb with well defined single layered epidermis. Arrows indicate trichome cells at the lower portion of plb. 40x.

Figure 79. Longitudinal section of developing plb showing apical meristem. 160x.

Figure 80. Longitudinal section of the tip of developing plb showing root initial (R) at the base of the shoot apex (S). 160x.

Figure 81. Medial longitudinal section of plb with shoot apex (S), root initial (R) and trichomes (T). 40x.

Figure 82. Longitudinal section of plb showing trichomes with cells containing dense cytoplasm and large nuclei. 160x.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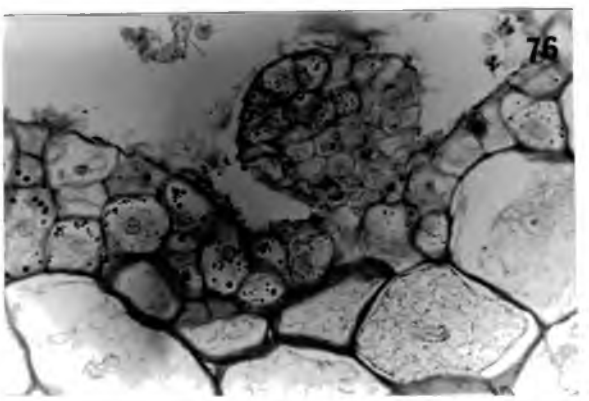
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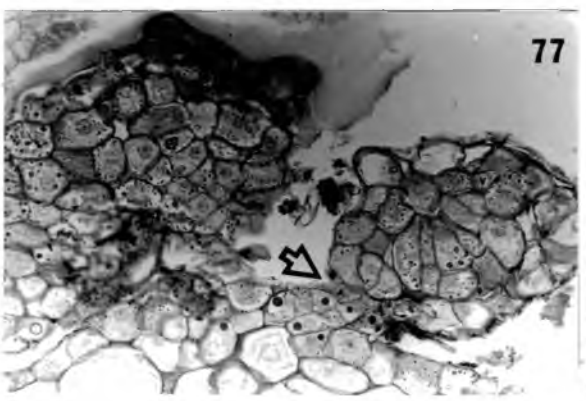
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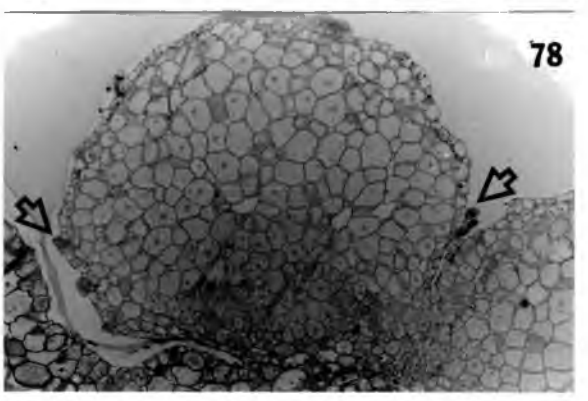
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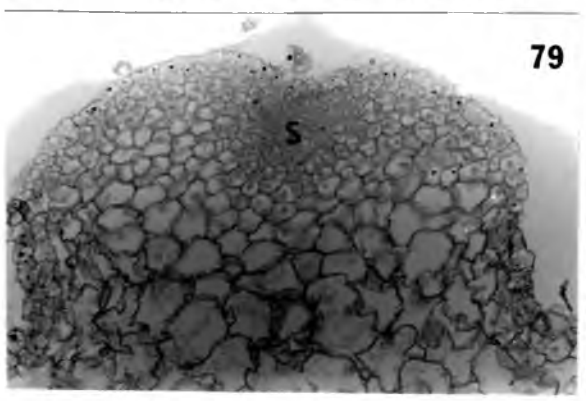
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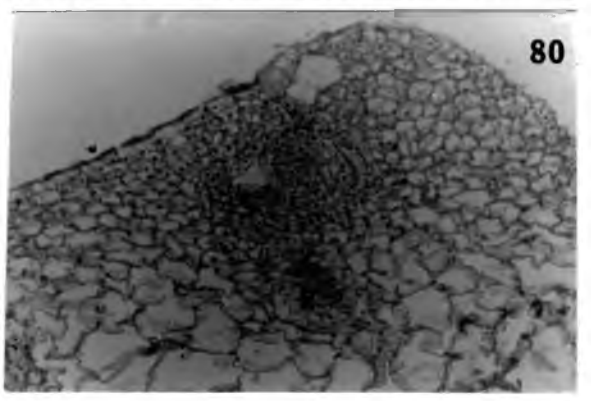
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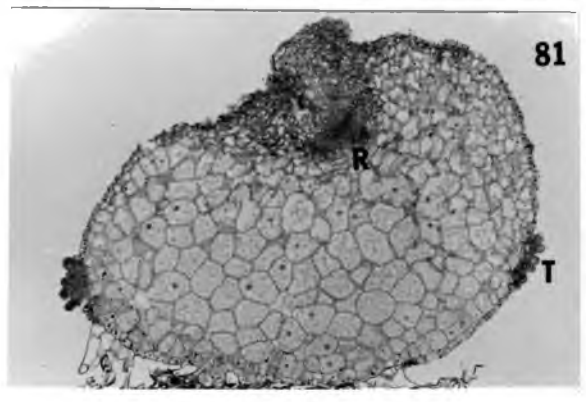
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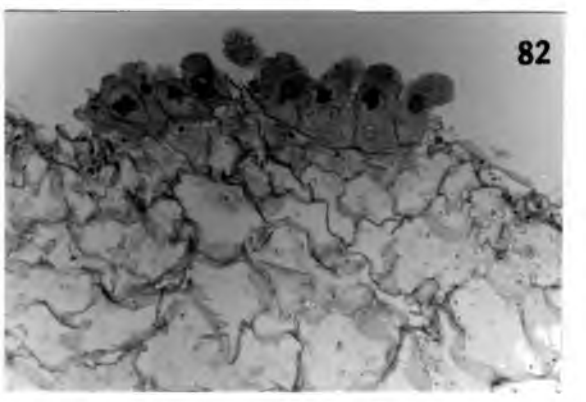

82

Formation and organization of plb on the epidermis of young leaf

Figure 83. Cross section of young leaf 1 month after subculture showing one active cell on the upper epidermis. 70x.

Figure 84. Cross section of the same leaf as in Figure 83 showing a group of meristematic cells on the epidermis. 50x.

Figure 85. Cross section of leaf showing two globular plbs. The one on the right is composed entirely of meristematic cells, and the one on the left of parenchymatous cells. 40x.

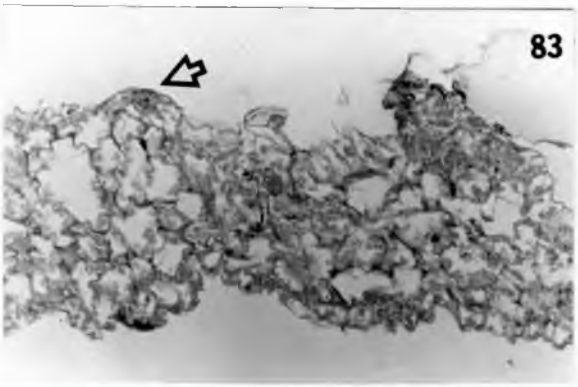
Figure 86. Longitudinal section of plb showing meristematic area which will form shoot apex. 160x.

Figure 87. Another cross section of leaf 1 month after subculture showing a few meristematic cells on the upper epidermis. 160x.

Figure 88. Cross section of leaf showing protuberances from the epidermal cells. 70x.

Figure 89. Close up of protuberance showing meristematic cells. 160x.

Figure 90. Cross section of leaf showing plbs developed on the protuberances from leaf epidermis. 40x.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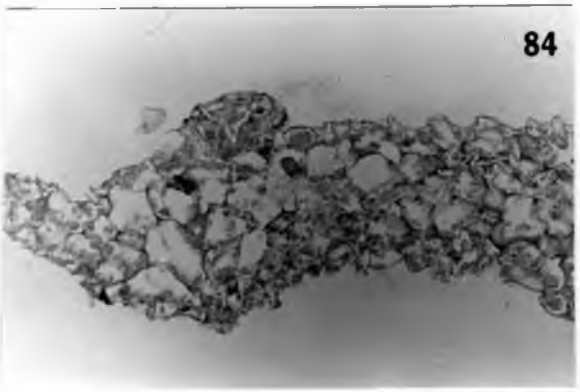
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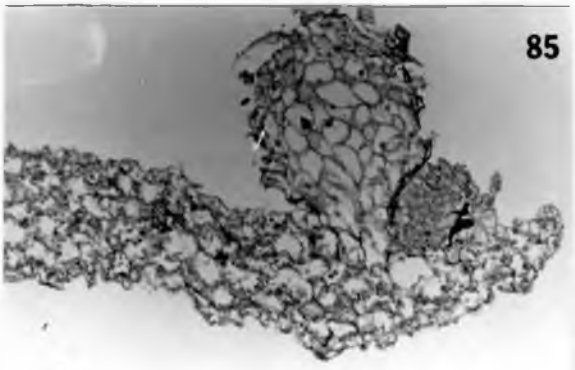
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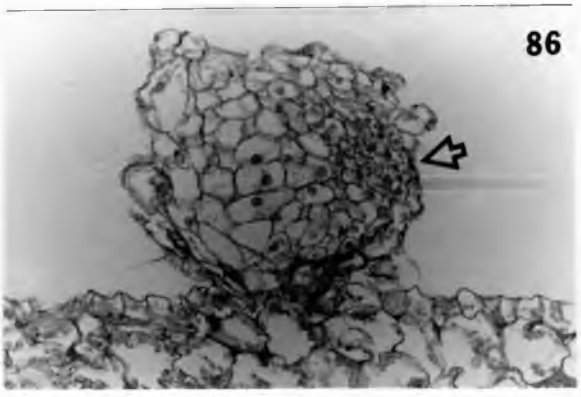
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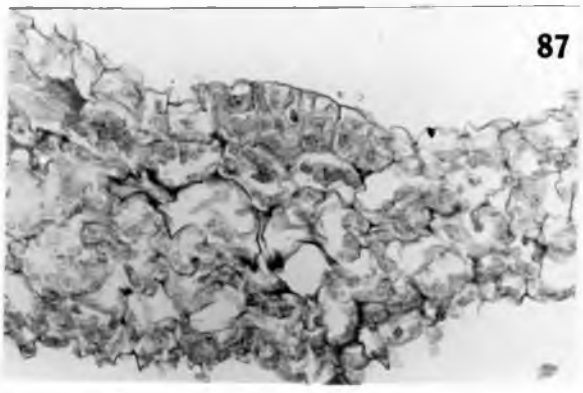
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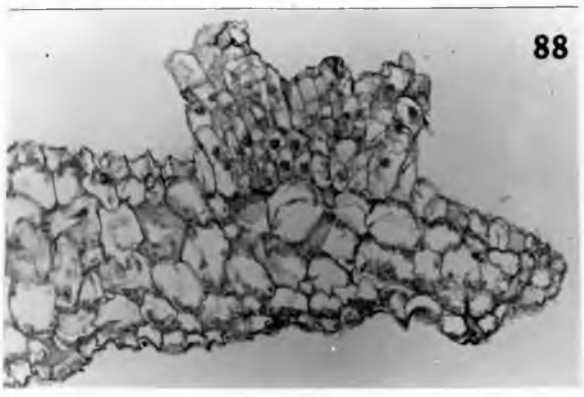
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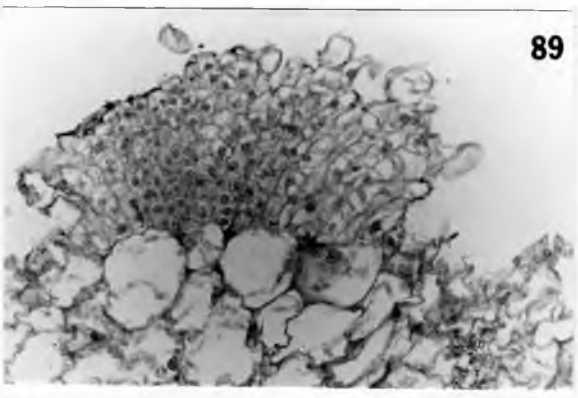
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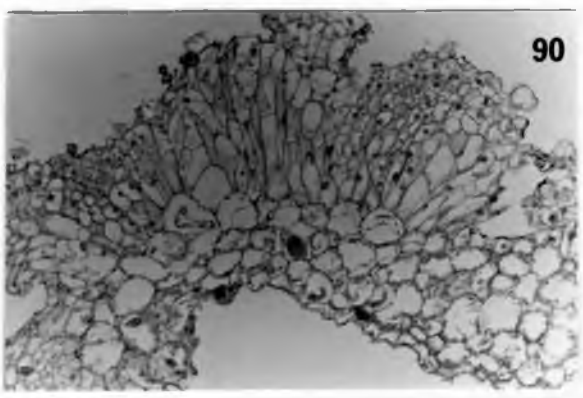
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DISCUSSION

Vegetative, asexual, or clonal propagation of Phalaenopsis is accomplished by division of plantlets which are induced by top cutting (Table II) or which form spontaneously on the tip of inflorescences (Phal. amabilis, Phal. intermedia, and Phal. equestris), on the node of inflorescences (Phal. lueddemanniana, Phal. cornu-cervi), on the tip as well as along the inflorescence (Phal. amabilis x Phal. lueddemanniana), or on the roots (Phal. stuartiana). The nature of spontaneous plantlet formation in Phalaenopsis and interspecific hybrids is not known. Phalaenopsis generally do not produce plantlets on the inflorescence. Usually during the flowering season, the buds on the inflorescence remain dormant and the tip continues to flower. If the inflorescence is cut just below the node which bore the first flower, only spikelets emerge from the upper nodes to produce a second spray of flowers (Northen, 1970).

Failure of vegetative buds on Phalaenopsis inflorescence to produce plantlets suggests an internal block of the growth process. The onset and termination of dormancy are apparently regulated by a balance of growth inhibitors and promoters (Walker, 1970). At the dormancy stage, the inhibitor-promoter balance favors the inhibitor components. This condition may be the result of either an excessive level of endogenous inhibitory substances such as phenolic compounds or abscisic acid (ABA) or the absence or deficiency of the promoters.

The application of cytokinins to axillary buds of apple (Chvojka et al., 1961; Williams and Stahly, 1968; Kender and Kapenter, 1972), peas and Helianthus sp. (Sachs and Thimann, 1964) overcomes apical dominance

and stimulates lateral bud growth. Also dormancy of buds of grape (Weaver, 1963) and peach (Weiberger, 1969) were terminated by treatment with cytokinin.

When N-6-benzyl adenine (BA) was applied to dormant buds on the inflorescence node after removal of bracts, shoot growth was initiated because the addition of an external growth promoter caused the inhibitor-promoter balance to shift in favor of promoters. Four types of growth were observed upon application of BA:

(1) The growth of the bud was stimulated during the first one or two months after which this bud became dormant again.

(2) The bud developed into multiple shoots but later became dormant. This is because the BA is depleted, or degraded by the environment, or leached by watering. The inhibitor-promoter balance shifted in favor of the inhibitors. In types one and two, the buds can be used as a source of explants in aseptic culture before they return into dormancy.

(3) The bud developed into a plantlet. This plantlet can be directly planted in the greenhouse.

(4) The bud formed a new inflorescence. The bud on the newly formed inflorescence could be treated repeatedly, causing more branching, so that more inflorescence nodes were suitable for use in inflorescence node propagation.

The type of growth of buds on the inflorescence node is dependent upon the hybrid, BA concentration, position on inflorescence and possibly season of the year. The application of BA at the lower nodes produced plantlets, whereas application at the upper nodes produced more inflorescence (Fig. 13). The level of flowering hormones was possibly

optimal near the tip. The tendency of producing plantlets at the lower nodes and inflorescences at the upper nodes is supported by *in vitro* experiments, which will be discussed later.

By application of BA *in vivo* it is possible to induce plantlets, but it is not recommended for commercial use, because only a small number of plantlets is obtained; however, it increases the number of plantlets that result from inflorescence node propagation by aseptic methods.

Heide (1965) found that cytokinin treatment of *Bryophyllum diagremontianum* leaves greatly increased the number of buds, but at the same time inhibited root formation. When BA was applied to *Phalaenopsis* buds both *in vivo* and *in vitro* (Figs. 15, 16, 41), multiple shoots with swollen base were obtained from a single node. This appears to be caused by mobilization of protein reserve food and photosynthetic products in the treated area. This mobilizing effect has also been reported in grapes (Letham, 1969, Quinlan and Weaver, 1969).

BA in lanolin paste is not translocated acropetally from the point of application (Fig. 10) and cannot penetrate through bracts (Fig. 9). To be effective in induction of plantlets, BA had to be applied on the bud. This method resulted in limited success because of the uncontrolled and unknown factors in the plants themselves or due to the influence of the environment. When these variables were eliminated by excision of the buds on the inflorescence aseptically culturing in BM + 5 ppm BA, plantlets were obtained.

In hybrids which remained dormant and eventually died there were probably growth inhibitors produced by the buds, and the growth promoters

such as gibberellin (Radley, 1958; Murashige, 1961), and cytokinins (Steward and Caplin, 1952b) from the coconut water could not counteract and overcome the inhibitors. Potato tissue has been found to possess inhibitory substances. The tissue requires 2,4-D in addition to coconut water to counteract the effect of inhibitory substances present in the tuber. Various other storage organs, dormant resting buds, and endosperms like those of caster bean, have all been shown to contain inhibitory substances (Steward and Caplin, 1952a). When a growth regulator such as BA at 1, 5, and 10 ppm was added to the medium, there was growth. The addition of 5 ppm BA caused rapid shoot formation in Phal. Chieftain x Phal. Zada (Fig. 35), and multiple shoot formation in Phal. Arcadia x Phal. cochleris (Fig. 36).

Some hybrids such as Phal. lueddemanniana x Phal. speciosa (1785) have grown on basal medium (BM) without addition of BA (Fig. 41). This is because the buds of these hybrids produced little or no growth inhibitors. The addition of BA to the media for culturing these hybrids resulted in yellowed leaves, slender elongated stems, and the formation of multiple shoots (Fig. 41). The effect of NAA for promoting root formation is well known. In Phalaenopsis inforescence node propagation, NAA has also been found to increase the number of roots formed and also inhibit shoot formation (Fig. 43).

When inflorescence nodes of 4 cm long of Phal. lueddemanniana x Phal. speciosa (1785) were cultured on BM medium, they grew into vigorous plantlets within 3 months. When inflorescence buds only were cultured on the same media, most of them remained dormant and died (Fig. 31). This indicated that buds produce growth inhibitors while inflorescence

stems store some growth promoters which minimize the effect of growth inhibitors.

Buds in different locations on the inflorescence produced different numbers of plantlets or inflorescences. Buds on the lower nodes showed a tendency to form plantlets while buds on the upper nodes produced inflorescences. Since the upper buds had been predetermined to become inflorescences at the time of cutting, inflorescences formed first. When the supply of flowering hormones was depleted, plantlets were produced at the tip or at the lowermost node of the inflorescences which had emerged.

The evidence from young inflorescence cultures has shown that 81.8% of the tip cultures produced dormant buds or died on medium with 5 ppm BA. This strongly indicated that the tip of the inflorescence produced inhibitors, or was the site of inhibitor accumulation at the time of cutting. The percentage of dormant buds or dead buds decreased basipetally. The further the distance from the tip, the lower the percentage of dead buds and the higher the percentage of plantlet formation (Table IX). These inhibitors are probably something other than the black exudate observed in culture. Results showed the tips produced less black exudate when compared to explants from position 3, 2 or 1 (Figs. 45, 46).

When plant tissues such as apple fruits or potato tubers are injured, the surfaces become brown (Salisbury, 1969). This browning reaction occurs because of the activity of phenol oxidase enzyme. Upon injury the enzymes apparently can contact certain phenolics such as catechol, chlorogenic acid, gallic acid, caffeic acid, or anthocyanin. The

hybroxyl groups of these phenolics are oxidized to quinone structures as shown in:

phenolics + oxygen ——————→ quinone group + water

Oxygen is absorbed in the process and is combined with the removed hydrogen to form water. The quinones can be further oxidized and polymerized, producing the complex colored substances which are responsible for the dark pigments often observed. This is found to inhibit growth. The polyphenol oxidase system appears to be present in Phalaenopsis. When inflorescences, terminal or axillary buds were cultured in vials, a black substance was produced by the explants which diffused into the medium within two or three days. Therefore, subculturing every ten days became necessary for liquid medium. The degree of blackness varied according to these factors:

Aeration: When cultured in agitated liquid medium, explants produced more black substance than in stationary culture. None of the initial explants produced plbs on the shaker. With other genera such as Cymbidium, Dendrobium, Cattleya, Oncidium, Vanda and Ascocenda, plbs were obtained in the agitated liquid media, and if cultured on solid media usually one or two plantlets were obtained. Therefore, agitation inhibits shoot formation and induces plb formation. Phalaenopsis had to be cultured in agitated liquid medium for one month to prevent shoot formation. Then the explants were transferred from the inhibitor environment to solid medium of the same formula. By so doing plbs were obtained on the plantlets, while the black substance diffused into the medium.

Anthocyanin: Purple-leafed species or hybrids produce more black substance than green-leafed species. This may be due to anthocyanin,

which is one of the precursors of the substance.

Plant part: Flower spikes produced more black substance than leaf and root.

Age: Plantlets produced more black substance than plb stage (Figs. 75, 72). The position of the node was also of importance; the lower nodes produced more black substance (Figs. 45, 46).

NAA inhibited elaboration of black substance.

BA promoted elaboration of black substance.

Ascorbic acid, maleic hydrazide, and tomato juice were used to reduce the elaboration of black substance, but it did not increase the success of plb formation. Some other chemicals such as cysteine, glutathione, polyvinyl pyrolidone and dithiothreital should be tried.

In conclusion, there are at least two kinds of inhibitors in Phalaenopsis. The inhibitor from tip or dormant buds is thought to be abscisic acid (ABA). The other kind of inhibitor is a black substance or phenol. Both kinds of inhibitors are involved in Phalaenopsis: these inhibit plb formation from the initial explants making Phalaenopsis one of the more difficult orchids to mericlone.

Inhibitors in Phalaenopsis have not yet been identified. The hypothesis that inhibitors were involved in bud dormancy was first suggested by Hemberg in 1949. These inhibitors came to be known as inhibitor β complex after Bennet-Clark and Kefford (1953). Components of inhibitor are reported to include toxic fatty acids (Bentley, 1958) abscisic acid (ABA) (Holst, 1971; Milborrow, 1967; Ryugo, 1969), and phenolic compounds such as cinnamic, ferulic, o-and p- coumaric acids (Varga, 1957; Varga and Koves, 1959), and salicylic acid (Holst, 1971).

Besides inhibitor β, other naturally-occurring inhibitors such as cyanides (Jones et al. 1957), and prunin (Erez and Lavee, 1969) have been isolated and were proposed to be implicated with the dormancy of plant organs. Perhaps inhibitors in Phalaeniosus include some of these chemicals.

Plbs were obtained from inflorescence node culture in Doritis pulcherrima and Doritaenopsis Red Coral (Figs. 25, 26), but mostly plantlets were produced in Phalaenopsis (Fig. 27). Within 3 months plantlets with 3 or 4 leaves and 1 or 2 roots were obtained. These plantlets were separated into leaf, stem and root, and cultured in specific media, producing additional plantlets.

Stem culture on BM medium produced 5 plantlets from axillary bud in 3 months. If these plantlets were re-excised and cultured every three months, 125 plantlets could be produced in a year starting from a single node.

Churchill, Ball and Arditti (1970) produced orchid plants from tissues excised from the leaf tips of seedlings. Champagnat (1969, 1970) obtained plantlets from leaf sheath of cattleya. In this study when leaves were cultured in agitated liquid media (BM + 50%CW - Su), for a month and then left in stationary liquid medium of the same formula, one to fourteen plantlets were obtained from single leaf cultures of Phal. John Seden, Phal. Arcadia x Phal. cochleris, and Dtn. Red Coral. Adventitious buds originated on the leaf sheath as shown in Figs. 47, 48 and arise from basal meristem as shown in Figure 50. In another case plantlets formed on the leaf base at the cut end (Fig. 49). This can be accounted for by some growth promoters in the leaves moving downward

and accumulating at the cut end, and activating the basal meristem to form adventitious buds.

Although in aseptic culture of orchids, media containing 2,4-D or other type of auxin have been used (Bergman, 1972), Kim (1973) reported for the first time that 2,4-D induced tumors which in turn give rise to limited numbers of plantlets. He found that the concentration of 0.1 ppm 2,4-D was too low to disturb the normal fluctuation of auxin in the root and normal growth continued. At 0.75 to 1 ppm 2,4-D tumor growth occurred in intact and excised roots. In this study with Phalaenopsis, 2,4-D at 0.5 to 1 ppm induced tumor growth on the excised stem, intact stem and excised root. Tumors originated and formed in a manner very similar to the formation of adventitious roots (Fig. 56) and branch roots (Fig. 59). Gorter and Zweep, 1964 reported that branch roots are induced by auxin. Therefore, it is possible that tumor growth and branch roots share the same origin from the pericycle. The initial growth is the same since they grow through the cortex and the epidermis. A major difference was the size of the meristematic zones. In the ordinary root tip as shown in Figure 60 there are meristematic cells which form the root cap,epidermis, cortex and vascular cylinder. These cells are active in a localized area near the tip of the root. Growth occurs in one direction--elongation resulting in a well defined longitudinal axis.

In most cases the tip of the root becomes swollen in 0.5 ppm 2,4-D medium, but later elongates as in a normal root. This suggests that the level of auxin is low enough not to permanently disturb the normal fluctuation of auxin in the root and therefore, normal root

elongation is not disturbed. At higher concentrations of 2,4-D (0.5 to 1 ppm) the normal regulation of elongation of root was disturbed, and tumors were formed characteristically. It can be concluded that the level of 2,4-D is important in determining whether or not root formation or tumor growth occurs.

In the normal condition the root cap degenerates as the root elongates. The root cap of the tumor also degenerated on 2,4-D medium. This tumor growth had to be transferred to 2,4-D free medium for plb formation and subsequent plantlet differentiation. By separating and subculturing the plbs, an indefinite number of plants were obtained.

Plbs could be obtained from shoot tip or inflorescence node culture. Plb can be easily obtained by shoot tip culture but in order to get terminal and axillary buds the mother plant has to be sacrified. Only two to four explants were obtained from a vegetative stem. Also a high percentage of contamination was obtained. Therefore, explants from the buds on the nodes of inflorescence were the most suitable material for culture. By doing this, plantlets were obtained instead of plbs. These plantlets were further re-excised and cultured in agitated liquid basal medium. Upon transfer to solid BM medium, plbs were obtained.

The medium for induction of plb is BM. Sucrose is the carbon source. Once plbs are formed, sucrose must be removed, to prevent yellowing of plbs. Coconut water has 2.17% sugars (Tulecke, 1961): therefore, BM contains about 0.32% sugar. Addition of 2% sucrose to this medium resulted in yellow plbs. It is suggested here that the high sucrose levels may lead to chlorophyll degradation as evidenced by chlorosis of plbs. This effect was also found in other monopodial types

such as Vanda, Ascocenda, Neofinetia, Rhynchostylis, Aranda, and in Dendrobium nobile. In sympodial types such as Cymbidium, Dendrobium, Cattleya and Oncidium, multiplication of plbs is best with addition of 2% sucrose to the medium. No severe chlorosis was observed. The differences in response of these two groups cannot be explained. Homes and Espen (1973) reported a reduction in chlorophyll content of Cymbidium plbs when the sucrose concentration was increased. Ultrastructural studies showed that chloroplasts contained large amounts of starch grains and the lamellar system was reduced. The chloroplasts became amylo-chloroplasts when the sucrose concentration was high. Ultrastructural studies on the chloroplast of the plbs of vandaceous types grown in sucrose medium should be pursued. Perhaps this sugar sensitive group have specific enzymes that can convert glucose and fructose to form starch granules in the chloroplast. As the starch grain grows bigger, it interupts the grana structure of the chloroplast, causing chlorosis. If the yellow plbs were transferred back to medium without sucrose, they became green again.

At plantlet stages since yellowing caused by sucrose symptoms are not found, perhaps photosynthates are translocated to be stored in the root or stem instead of in the form of starch grain in the leaf plastid. Both monopodial and sympodial plantlets grow well in sucrose medium. In some species sucrose is necessary for root formation. Lindemann et al. (1970) showed that upon addition of 2% sucrose in medium for culturing Cattleya, roots formed within 10 days.

Aseptic cultures of shoot tip, inflorescence node, stem, leaf and root of Phalaenopsis formed a variety of organs - shoots, flowers, roots, and protocorm-like bodies. The most frequent type of regeneration is

shoot formation. Many plant tissues-chicory roots (Camus, 1949), carrots (Steward et al., 1958), potatoes (Fellenbert, 1963), and Convolvulus (Bonnet & Torrey, 1966) have been reported to produce shoots of normal organization.

The anatomical events during the induction and development of shoots were first described by Sterling (1951). Shoots arise in tobacco stalk culture from elements of the external phloem. In chicory from the cambium (Camus, 1949) and in Convolvulus roots from the cells near the protoxylem (Bonnet & Torrey 1966). Reinert (1973) concluded that the origin of shoots may be endogenous or exogenous depending on the plant. Both types were reported in Convolvulus callus. In this study it was found that buds formed on the inflorescence node section arose exogenously (Fig. 32), and also adventitious buds arose from the tissue of leaf sheath (Fig. 50).

Inflorescence node culture of Doritis (1669) produced flowers under aseptic condition as shown in Figure 28. Flower primordia in vitro and their subsequent development to complete flowers were first observed in explants of stalks of tobacco (Aghion-Prat, 1965). Segments from different parts of the stalks of flowering plants behaved differently; at the older basal internodes only vegetative buds were produced, while flower buds formed on the young upper parts, particularly on explants from the inflorescence. These findings were confirmed by the experiment with inflorescence node cultures of Doritis (1669) (Figs. 37 to 40). The lower the position of the node, the higher the number of plantlets formed; the higher the node the greater the tendency to form inflorescences (Table VIII).

One of the leaf cultures of Dtn. Red Coral (1237) produced roots followed by shoot as shown in Figure 47. Roots were first observed by Nobecourt (1939) in cultures from carrots. Later there followed many reports that mentioned root formation from callus as well as in cell suspension cultures. These include woody plants (Jaquiot, 1951), parenchyma from tobacco shoots (Skoog and Miller, 1957), and pea roots (Torrey, 1959). The origin of roots in callus culture are from nodule (Steward et al., 1952). Normally they are distributed irregularly over the surface of the callus and, in suspensions, can grow on various components (Reinert, 1973).

In this experiment plbs, and shoots were produced from shoot tip, inflorescence node and organ cultures. The question raised was what is the criteria for protocorm-like bodies, to be distinguished from developing buds? The criterian used here was the presence of trichomes (rhizoids) on plbs. Haccius (1971) stated that the decisive feature for categorizing a plant structure as an embryo, besides other morphological properties, is its bipolarity and the fact that in the earliest development stage, it has a shoot and a radicular pole at opposite ends. Furthermore, this system must not be connected with the vascular tissue of the mother plant or the explant during its initiation and development. With monopolar buds and roots, on the other hand, it is always possible to show their connection with the vascular elements of the mother plant or in the callus.

Plbs in Phalaenopsis were obtained in shoot tip culture where the size of explants is small. Perhaps there are less vascular elements in the shoot tip, whereas in inflorescence node cultures, mostly shoots were

formed and there was connection between vascular bundle of stem to the bud (Fig. 32). The resulting plantlets from leaf cultures were also shown in Figure 50, where plbs were obtained from the first leaf (Figs. 81, 82). Shoot or plbs formation on leaf is probably influenced by the vascular tissue in the original explants.

Reinert (1958, 1959) described the sequential development from pro-embryonal, globular and torpedo through embryo stage in carrot root tissue culture. There are many later reports which confirm the formation of embryoids in callus or cells of carrot (Kato & Takeuchi, 1963; Steward et al., 1964; Halperin 1966a; and Steward, et al., 1970), Solanum (Yamada et al., 1967); Atropa spp. (Konar et al., 1972a). It has been speculated that embryos in tissue cultures are formed from single cells, usually at the upper surface of the callus. The situation is similar to embryo formation in callus of Ranunculus. The majority of embryos originated from single cells at the surface of the callus (Konar et al., 1972b). Similar observations have been made on suspensions containing single cells and cell aggregates (Steward et al., 1966). The isolated single cells develop directly by segmentation without callus formation into pro-embryos globular, a torpedo, and embryo to normal plantlets (Steward et al., 1970).

The young inflorescence of Ascofinetia Cherry Blossom was induced to proliferate and form plantlets (Intuwong and Sagawa, 1972). The epidermal cells all along the surface of the inflorescence axis showed meristematic activity, formed protruding cells and eventually developed into plbs. The tissue from which plbs originate varied a great deal with genera and species (Intuwong, 1972). Morel (1971) reported that

sometimes the outgrowth came from the central axis of leaf primordia in Cymbidium, or in Cattleya, the axis remained inert and only the cells of the leaf epidermis proliferated. In the case of Vanda the cortex of the stem near the apex proliferated. In the case of inflorescence culture of Neofinetia, the outgrowth came from the epidermis of the inflorescence axis. In the apical and axillary bud cultures of Sarcanthine orchids, outgrowths originated from the cut surface of the scale-like leaves in the nodal area. Also in some cultures, young differentiated leaves proliferated. In Phalaenopsis the origin of plbs from the original explants is unknown. In the study of plbs from the old plb after isolation it was shown that one cell in the epidermal layer is active, divided, anticlinally elongated out of the surface, and further divided periclinally as well as anticlinally to form a globular structure. The cells continued to divide in certain area, and enlarged in the other. The meristematic region formed a shoot tip. After the formation of shoot tips, the root initial was observed deep in the tissue just under the shoot apex.

SUMMARY

Clonal propagation of *Phalaenopsis* can be accomplished *in vivo* by division of plantlets spontaneously formed on the inflorescence node, inflorescence tip, or root in some of *Phalaenopsis* species and hybrids. Plantlets can also be induced by top cutting or application of 2000 ppm N-6-benzyl adenine (BA) in lanolin paste on dormant bud of the lowermost node of inflorescence after removal of bract.

In vitro shoot tip cultures of terminal and axillary buds, and dormant buds on inflorescence node induced by 2000 ppm BA before excision produced plantlets or protocorm-like bodies in basal medium (BM = Vacin and Went + 15% coconut water) which developed into plants.

Inflorescence nodal cultures from mature inflorescences on BM either remained or produced plbs, plantlets and inflorescences. BA at 5 ppm promoted rapid shoot formation, and at 10-20 ppm caused multiple shoot formation but inhibited root formation. α-Naphthaleneacetic acid (NAA) at 1-10 ppm promoted a number of roots but inhibited shoot formation.

Tip and nodal cultures of young inflorescences in BM + 5 ppm BA either remained dormant or produced plbs, plantlets and inflorescences. Most of the tip cultures died.

Additional plantlets were produced by culturing the main axis or leaves of plantlets in sterile culture. Intact or excised roots or stems cultured on BM + 1 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) produced tumor growths which later produced a small number of plbs and plantlets.

For optimum growth, plbs required different media depending on the stage of development--for induction of proliferation of plb on explant agitated liquid medium of BM for one month and transfer to agar medium of the same formula, for multiplication and differentiation of plbs into plantlets agar medium of BM - Su, and for growth of plantlets basal agar medium.

Production of plbs were obtained by subculturing plb at one leaf stage. Usually a cell in the epidermis of original plb or young leaf divided anticlinally forming two protruding cells. Further divisions of these meristematic cells formed a globular plb. Cells on one side of the globular structure enlarged while cells in the other portion remained meristematic. This meristematic area organized a shoot tip before root initial.

LITERATURE CITED

Aghion-Prat D. 1965. Neoformation de fleurs in vitro chez Nicotiana tabacum L. Physiol. Végét, 3:229-303.

Bennet-Clark, T. A. and N. P. Kefford. 1953. Chromatography of the growth substances in plant extracts. Nature 171:645-649.

Bentley, J. A. 1958. The naturally occurring auxins and inhibitors. Ann. Rev. Plant Physiol. 9:47-80.

Bergman, F. L. 1972. Shoot tip multiplication of orchid clones, Part II. Amer. Hort. 51:41-44.

Bertsch, W. 1967. A new frontier: Orchid propagation by meristem tissue culture. Am. Orchid Soc. Bull. 36:32-37.

Bonnet H. T. and Torrey, J. G. 1966. Comparative anatomy of endogenous bud and lateral root formation in Convolvulus arvensis roots cultured in vitro. Am. J. Bot. 53:496-507.

Camus, G. 1949. Recherches sur le rôle des bourgeons dans les phénomènes de morphogénèse. Rev. Cytol. Biol. Vég. 11:1-195.

Champagnat, M. and G. Morel. 1969. Multiplication végétative des Cattleya a patir de bourgeons cultivés in vitro. Soc. bot. Fr., Memoires. 111:132.

_____, _____, and B. Mounetou. 1970. La multiplication végétative des Cattleya a patir de jeunes feuilles cultivés aseptiquement in vitro. Ann. Sci. Natur. Ser. 12(XI):97-114.

Churchill, M. E., E. A. Ball, and J. Arditti. 1970. Production of orchid plants from seedling leaf tips. Orchid Dig. 34:271-273.

_____, J. Arditti, and E. A. Ball. 1971. Clonal propagation of orchids from leaf tips. Am. Orchid Soc. Bull. 40:109-113.

_____, E. A. Ball, and J. Arditti. 1973. Tissue culture of orchids. I. Methods for leaf tips. New Phytologist. 72:161-166.

Chvojka, L., Veres, K., and Kozel, J. 1961. The effect of kinins on the growth of apple-tree buds and on incorporation of radioactive phosphate. Biol. Plant. 3:140-147.

Erez, A. and S. Lavee. 1969. Prunin identification, biological activity and quantitative change in comparison to naringenin in dormant peach buds. Plant Physiol. 44:342-326.

Fast, G. 1973. Die vermehrung von Oncidium papilio durch triebspitzen kultur und besprechung einiger nährmedien. Die Orchidee 24:240-246.

Fellenberg, G. 1963. Über die organbildung an in vitro kultiviertem knollengewebe von Solanum tuberosum. Z. Bot. 51:113-141.

Fonnesbech, M. 1972a. Growth hormones and propagation of Cymbidium in vitro. Physiol. Plant. 27:310-316.

_____. 1972b. Organic nutrients in the media for propagation of Cymbidium in vitro. Physiol. Plant. 27:36-364.

Gorter, C. J. and W. V. O. Zweep. 1964. Morphogenetic effect of herbicides. In : Physiology and biochemistry of herbicides. L. J. Audus, ed., New York, Academic Press. pp. 135-275.

Haccius, B. 1971. Zur derzeitigen situation der Angiospermen-Embryologie. Bot. Jb. 91:309.

Halperin, W. 1966a. Alternative morphogenetic events in cell suspensions. Am. J. Botany. 53:443-453.

_____. 1966b. Single cells, coconut milk and embryogenesis in vitro. Science. 153:1287-1288.

Heide, O. M. 1965. Effects of 6-Benzylaminopurine and 1 naphthaleneacetic acid and epiphyllous bud formation in Bryophyllum. Planta 67:281-296.

Homes, J. and N. V. Espen. 1973a. Effects du saccharose et de la lumiere sur le développement et la morphologie de protocormes d'orchidées cultivés in vitro. Bull. Soc. Roy. Bot. Bel. 106:89-106.

_____, and _____. 1973b. Quelques formes de plastes induites par le milieu de culture dans des protocormes d'orchidees cultives in vitro. Bull. Soc. Roy. Bot. de Bel. 106:117-121.

Holst, U. B. 1971. Some properties of inhibitor from Solanum tuberosum compared to abscisic acid. Physiol. Plant. 24:392-396.

Intuwong, O. 1972. Meristem culture of miniature monopodial orchids. M.S. Thesis, Univ. of Hawaii: 68 pp.

_____, J. T. Kunisaki, and Y. Sagawa. 1972. Vegetative propagation of Phalaenopsis by flower stalk cuttings. Na Okika O Hawaii. 1(4):13-18.

_____, and Y. Sagawa. 1973. Clonal propagation of Sarcanthine orchids by aseptic culture of inflorescences. Am. Orchid Soc. Bull. 42:209-215.

Jacquiot, C. 1951. Action du meso-inositol et de l'adenine sur la formation de bourgeons par le tissu cambial. C. r. hebd, Seanc. Acad. Sci., Paris 233:815-817.

Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill. New York. 523 pp.

Sterling, C. 1951. Origin of buds in tobacco stem segments cultured in vitro. Am. J. Bot. 38:761-767.

Steward, F. C., P. V. Ammirato, and M. O. Mapes. 1970. Growth and Development of totipotent cells : some problems, procedure, and perspective. Ann. Bot. 34:761-787.

_____, S. M. Caplin. 1952a. Investigations on growth and metabolism of plant cells. III. Evidence for growth inhibitors in certain mature tissue. Ann. Bot. 16:477-489.

_____, _____. 1972b. Investigations on growth and metabolism in plant cells. IV. Evidence on the role of the coconut-milk factor in development. Ann. Bot. 16:491-504.

_____, A. E. Kent, and M. O. Mapes. 1966. The culture of free plant cells and its signification for embryology and morphogenesis. Current Topics in Developmental Biology. Academic Press. New York. 113-154.

_____, M. O. Mapes, A. E. Kent, and R. D. Holsten. 1964. Growth and development of cultured plant cells. Science. 143:20-27.

_____, _____, and K. Mears. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Ann. J. Bot. 45:705-708.

Teo, C. K. H., J. T. Kunisaki, and Y. Sagawa. 1973. Clonal propagation of strap-leafed vanda by shoot-tip culture. Am. Orchid Soc. Bull. 42:402-405.

Thompson, N. P. and W. P. Jacobs. 1966. Polarity of IAA effect on sieve-tube and xylem regeneration in Coleus and tomato stems. Pl. Physiol. 41:673-682.

Torrey, J. G. 1959. Experimental modification of development in the root, in Cell, Organism and Milieu. Ed. D. Rudnick Ronald Press. New York. 189-222.

Jones, M. B., J. W. Fleming and L. F. Bailey. 1957. Cyanide as a growth-inhibiting substance in extracts of peach leaves, bark, and flower buds. Proc. Amer. Soc. Hort. Sci. 69:152-157.

Kato, H. and M. Takeuchi. 1963. Morphogenesis in vitro starting from single cells of carrot roots. Pl. Cell Physiol. 4:243-245.

Kender, W. J., and S. Carpenter. 1972. Stimulation of lateral bud growth of apple tree by 6-benzylaminopurine. J. Amer. Soc. Hort. Sci. 97:377-380.

Kim, K. K. 1973. Studies on the response of orchid roots to 2,4-dichlorophenoxyacetic acid (2,4-D). Ph.D. Dissertation, Univ. of Hawaii. 97 pp.

Konar, R. N., E. Thomas, and H. E. Street. 1972a. The diversity of morphogenesis in suspension cultures of Atropa belladonna L. Ann. Bot. 36:123-145.

_____, _____, and _____. 1972b. Origin and structure of embryoids arising from epidermal cells of the stem of Ranunculus sceleratus. L. J. Cell Sci. 11:77-93.

Kotomori, S., and T. Murashige. 1965. Some aspects of aseptic propagation of orchids. Am. Orchid Soc. Bull. 34:484-489.

Kunisaki, J. T., K. K. Kim, and Y. Sagawa. 1972. Shoot-tip culture of vanda. Am. Orchid Soc. Bull. 41:435-439.

Letham, D. S. 1969. Cytokinins and their relation to other phytohormones. Bio. Sci. 19:309-316.

Lindemann, G. E. P., J. E. Gunckel, and O. W. Davidson. 1970. Meristem culture of Cattleya. Am. Orchid Soc. Bull. 39:1002-1004.

Milborrow, B. V. 1967. The identification of (+) - abscisin II (+) - dormin in plants and measurements of its concentrations. Planta 76:93-113.

Morel, G. M. 1960. Producing virus-free Cymbidium. Am. Orchid Soc. Bull. 29:495-497.

_____. 1964. Tissue culture : a new means of clonal propagation of orchids. Am. Orchid Soc. Bull. 33:473-478.

_____. 1965. Clonal propagation of orchids by meristem culture. Cymbidium Soc. News. 20:3-11.

_____. 1971. The principles of clonal propagation of orchids. Proc. Sixth World Orchid Con. 101-106.

Murashige, T. 1961. Suppression of shoot formation in cultured tobacco cells by gibberellic acid. Science. 134(3474):280.

Nobecourt, P. 1939. Sur les radicelles naissant des cultures de tissus vegetaux. C. r. Seanc. Soc. Biol. 130:1271.

Northen, R. T. 1970. Home Orchid Growing. 3rd. Edition. D. Van Nostrand Co., Inc., Princeton, New Jersey. 374 pp.

Quinlan, J. D., and R. J. Weaver. 1969. Influence of benzyladenine, leaf darkening, and ringing on movement of ^{14}C-labeled assimilates into expanded leaves of Vitis vinifera P. Plant Physiol. 44:1247-1252.

Radley, M. 1958. The distribution of substances similar to gibberellic acid in higher plants. Ann. Bot. N. S. 22:297-307.

Reinert, J. 1958. Untersuchungen über die morphogenese an gewebekulturen, Ber. dtsch. Bot Ges. 71:15.

_____. 1959. Über die Kontrolle der morphogenese und die induktion von anventivembryonen an gewebekulturen aus karotten. Planta. 53:318-333.

_____. 1973. Aspects of organization-Organogenesis and embryogenesis. In Plant Tissue and Cell Culture. H. E. Street, Univ. of California Press, Berkeley. 503 pp.

Reinert, R. A. and H. C. Mohr. 1967. Propagation of Cattleya by tissue culture of lateral bud meristems. Proc. Am. Soc. Hort. Sci. 91:664-671.

Rotor, G. 1949. A method of vegetative propagation of Phalaenopsis species and hybrids. Am. Orchid Soc. Bull. 18:738-739.

Ryugo, K. 1969. Abscisic acid, a component of the beta-inhibitor complex in the prunus endocarp. J. Amer. Soc. Hort. Sci. 94:5-8.

Sachs, T. and K. V. Thimann. 1964. Release of lateral buds from apical dominance. Nature 201:939-940.

Sagawa, Y. and D. Niimoto. 1960. Vegetative propagation of Phalaenopsis. Florida Orchidist. 3(5&6):22.

_____. 1961. Vegetative propagation of Phalaenopsis stem cuttings. Am. Orchid. Soc. Bull. 30:808-809.

_____, T. Shoji and T. Shoji. 1966. Clonal propagation of Cymbidiums through shoot meristem culture. Am. Orchid Soc. Bull. 35:118-122.

_____, _____, and _____. 1967. Clonal propagation of Dendrobiums through shoot meristem culture. Am. Orchid Soc. Bull. 36:856-859.

Scully, R. M. 1965. Stem propagation of Phalaenopsis. Bull. Pac. Soc. of Hawaii. 23:13-16.

_____. 1966. Stem propagation of Phalaenopsis. Am. Orchid Soc. Bull. 35:40-52.

_____. 1967. Aspects of meristem culture in the Cattleya alliance. Am. Orchid Soc. Bull. 36:103-108.

Salisbury, F. B., and R. Cleon. 1969. Plant Physiology. Wadsworth Publishing Company, Inc. Belmont, Calif. 747 pp.

Shapiro, S. 1947. Warm saffranin for plant tissues. Science. 105:50.

Skoog, F. and C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. exp. Biol. 11:118-130.

Tse, Anthony Tin-Yaw, R. J. Smith, and W. P. Hacket. 1971. Adventitious shoot formation on Phalaenopsis nodes. Amer. Orchid Soc. Bull. 40:807-810.

Tulecke, W., L. H. Weinstein, A. Rutner, and H. J. Laurencot. 1961. The biochemical composition of coconut water (coconut milk) as related to its use in tissue culture. Contrib. Boyce Thompson Inst. 21:115-128.

Urata, U. and E. T. Iwanaga. 1965. The use of Ito-type vials for vegetative propagation of Phalaenopsis. Am. Orchid Soc. Bull. 34:410-413.

Vacherot, M. 1966. Meristem tissue culture propagation of orchids. Proc. Fifth World Orchid Conf. 23-26.

Vajrabhaya, M. and T. Vajrabhaya. 1970. Tissue culture of Rhynchostylis gigantea, a monopodial orchid. Am. Orchid Soc. Bull. 39:907-910.

Varga, M. 1957. Examination of growth-inhibiting substance separated by papar chromatography in fleshy fruits. II. Identification of the inhibiting substance of the chromatograms. Acta Biol. Szeged. 3:312-323.

_____, and E. Koves. 1959. Phenolic acids as growth and germination inhibitors in dry fruits. Nature 183:401.

Walker, D. R. 1970. Growth substances in dormant fruit buds and seeds. Hort. Sci. 5:414-417.

Weaver, R. J. 1963. Use of kinin in breaking rest of buds of Vitis vinifera. Nature 198:207-208.

Weiberger, J. H. 1969. The stimulation of dormant peach buds by a cytokinin. Hort. Science. 4:125-126.

Werckmeister, P. 1971. Light induction of geotropism and the control of proliferation and growth of cymbidium in tissue culture. Bot. Gaz. 132(4):346-350.

Williams, M. W., and Stahly, E. A. 1968. Effect of cytokinins on apple shoot development from axillary buds. Hort. Science 3:68-69.

Wimber, D. E. 1963. Clonal multiplication of cymbidiums through tissue culture of the shoot meristem. Am. Orchid Soc. Bull. 32:105-107.

_____. 1965. Additional observations on clonal multiplication of cymbidiums through culture of shoot meristem. Cymbidium Soc. News. 20(4):7-10.

Yamada, T., Nakagawa, and Shinobo, Y. 1967. Studies on the differentiation in cultured cells. I. Embryogenesis in three strains of Solanum callus. Bot. Mag. Tokyo. 80:68-74.